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1. Your reference

M98/0599/GB

2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Tepnel Medical Limited
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Patents ADP number *(if you know it)*

4446954002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Enzymatically Catalysed Signal Amplification

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

McNeight & Lawrence
Regent House, Heaton Lane
Stockport, Cheshire SK4 1BS

Patents ADP number *(if you know it)*

0001115001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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Request for substantive examination *(Patents Form 10/77)*	
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11. I/We request the grant of a patent on the basis of this application.

Signature Haight Lawrence Date 10/03/99

12. Name and daytime telephone number of person to contact in the United Kingdom James A Robertson 0161-480-6394

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Enzymatically Catalysed Signal Amplification.

The present invention concerns detection of a target molecule by novel methods of enzymatically catalysed amplification of target associated detectable structures.

As used herein the term "nucleic acid" includes protein nucleic acid (PNA) (i.e. nucleic acids in which the bases are linked by a polypeptide backbone) as well as the naturally occurring nucleic acids (e.g. DNA and RNA), or analogues thereof, having a sugar phosphate backbone.

Several nucleic acid amplification techniques are already known, e.g. the Polymerase Chain Reaction (PCR). However many of these techniques (including PCR) suffer from the disadvantage that they specifically amplify a target sequence (amplicon) present within the sample of interest. This amplicon, once generated, can easily contaminate a laboratory working area in which strict controls are not maintained. Such contamination can render subsequent amplification reactions suspect, and can require a cessation of testing and the initiation of expensive decontamination procedures.

Techniques such as PCR, which detect the presence of a sequence by amplifying its number to detectable levels, are known as Target Amplification systems. In contrast, several techniques are known which amplify a signal to detectable levels, usually following the binding of a detector molecule to the molecule of interest, *without* amplification of said molecule. These are referred to as Signal Amplification techniques.

One such system, held under patent by Chiron Corporation (Urdea et al., see patent, 5681697 and refs. cited therein) is known as the branched DNA system (bDNA). This system relies on the binding to the target molecule, of a large number of detector probes, which contain within their sequence a site for hybridisation of a non-target specific sequence. This sequence acts as the site for hybridisation of a pre-synthesised branched DNA structure with numerous sites for the hybridisation of secondary branches or of detection probes. One of the main disadvantages of this system is the large number of components required to generate the final structure to which the detection probes adhere. In addition, the many hybridisation steps involved in the assembly process render the system susceptible to the production of non-specific background signal.

The current invention outlines a method for the amplification of a nucleic acid based signal. It involves the generation of a repeating structure containing multiple copies of a detectable sequence, and is produced through the concerted action of a polymerase (which extends the repeating structure) and a separating agent (which uncovers hybridisation sites to

allow assembly of sequence repeats). The method of the invention offers the following advantages over methods existing in the art:

1. It uses a small number of inexpensive components and so avoids the high cost per assay problems associated with other signal amplification systems such as bDNA.

2. It uses a signal amplification rather than a target amplification approach and thus it overcomes contamination problems associated with many current methodologies such as PCR.

3. It is enzymatically catalysed process that actively assembles the signal generating structure rather than relying on the passive hybridisation-based approaches of non-enzymatic methods such as bDNA.

4. It has the ability to address RNA and DNA targets with equal efficiency without pre-treatments, unlike for example PCR which requires an initial reverse transcription step before RNA targets can be amplified.

5. As the process is used in conjunction with a solid phase immobilised target it has the potential to be integrated into 'cutting edge' solid phase devices such as Biochips.

DESCRIPTION OF THE INVENTION.

It is envisaged that the target molecule may first be immobilised on a solid phase using methods known in the art before application of the amplification system but the invention is not limited to this or any other specific assay format. It will also be understood that the descriptions of the embodiments are used for illustrative purposes only and are not intended to be limiting. Some of the basic components and properties of the system are outlined below;

i) Locator probe.

The probe contains two distinct regions, the target binding moiety and the amplification moiety. The function of the target binding moiety is to locate to and interact with the target molecule so as to become located at that point. The target binding moiety may comprise anything capable of specifically binding the target molecule. For example in the case of the target molecule being a nucleic acid sequence, the binding moiety may comprise a nucleic acid sequence complementary to the target nucleic acid sequence. Alternatively, it may comprise RNA, a mixture of RNA and DNA or for example PNA. Alternatively, the binding moiety may

comprise an antibody or an antigen binding fragment thereof specific to the target molecule (Harlow, E. and Lane, D., "Using Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1998). Other binding mechanisms (for example involving covalent bonding) and reagents will be readily apparent to one skilled in the art. The amplification moiety comprises a nucleic acid sequence that is non-complementary to the target and should not bind to it, therefore rendering it accessible for later binding events. Where the target molecule is a nucleic acid sequence, the Locator probe could be so constructed that its target binding moiety and signal moieties were oligonucleotides joined using a 5'-3' linkage, or alternatively using a 5'-5' linkage such that each region possessed an extendible free 3' end. Where the target binding moiety is an antibody or antigen binding fragment, the amplification nucleic acid moiety may be covalently bound to it using methods known in the art (see, for example, Ito, W. and Kurosawa, Y., 1993, Journal of Biological Chemistry, 268(27): 20668-20675).

ii) **Primary amplification template and the Primary Structure.**

The amplification template is most frequently a single stranded nucleic acid sequence comprising, arranged in a 5'-3' direction, an extension region, a hybridisation region and an amplification moiety. It is designed to hybridise via its hybridisation region to a complementary nucleic acid sequence. Under the appropriate reaction conditions the 3' end of this hybridised complement can be extended to create a complement to the extension region of the template. Selective removal of the extension region of the template then leaves a newly synthesised single stranded region to which an additional amplification template can hybridise. A repetition of the steps of hybridisation, extension and removal results in an extended structure composed of multiple repeats of the template. To distinguish this structure from others which may be subsequently generated it is referred to as the Primary Structure. The amplification templates involved in its construction are referred to as primary amplification templates.

Within the Primary Structure are the amplification moieties of the incorporated primary amplification templates. These regions, being non-complementary to the target or Primary Structure, are available to be hybridised with appropriately labelled Detection probes.

If the hybridisation region of the primary amplification template was designed for example to be complementary to the amplification moiety of the Locator probe, then the Primary Structure would be attached to the Locator probe which itself would be attached to the target. Hybridisation of the Detection probes to the Primary Structure would therefore provide a linearly amplified number of signal generating sites linked to the target sequence.

iii) **Secondary amplification templates and the Secondary Structure.**

As an alternative to providing hybridisation sites for Detection probes, the primary template amplification moieties incorporated into the Primary Structure can act as complements to the hybridisation region of secondary amplification templates. Secondary amplification templates are most frequently single stranded nucleic acid sequences comprising, arranged in a 5'-3' direction, an extension region, a hybridisation region and a amplification moiety.

The hybridisation region can be so designed to be complementary to the amplification moiety of the primary amplification template. Under the appropriate reaction conditions the 3' end of the primary template amplification moiety can be extended to create a complement to the extension region of the secondary template. Selective removal of the extension region of the secondary template then leaves a newly synthesised single stranded region to which an additional secondary amplification template can hybridise. A repetition of the steps of hybridisation, extension and removal results in multiple extended structures (composed of multiple repeats of the secondary template) attached to the Primary Structure. This structure is referred to as the Secondary Structure.

The incorporated amplification moieties of the secondary templates being non-complementary to the target, Primary Structure or Secondary Structure are available to be hybridised to by appropriately labelled Detection probes. Overall a non-linear increase in the number of target associated signal generating sites is provided by the generation of the Secondary structure.

Each reaction, which results in the assembly of a structure, is referred to as a repeat. The first repeat generates the Primary Structure, the second repeat the Secondary structure and so on. It will be clear to those practiced in the art that Tertiary and Quaternary structures etc., could be generated in this fashion by carrying

out the appropriate number of repeats. The number of repeats carried out will depend on the application and will reflect the sensitivity required.

iv) **Separation Agents.**

The generation of the extended Primary or Secondary structure etc., relies on the removal of the extension region of the amplification template once the complement to that region has been made. The 'uncovering' of this newly synthesised strand provides a site for another amplification template to hybridise and thus continue the assembly process. Separation agents are responsible for removal of the extension region. These separation agents can include any one or combination of the following; a 5' double strand specific exonuclease (such as T7 gene 6 exonuclease, or Lambda exonuclease); a restriction endonuclease; an RNase (such as RNase H); elevated temperature; or chemical denaturation.

Selective removal of the extension region, whilst ensuring the other regions of the incorporated amplification template remain attached to the growing structure requires some means of controlling or limiting the action of the separating agents. Protection against exonuclease may be achieved through the use of nucleotide analogues or synthetic nucleic acid sequences (e.g., phosphorothioate linkages or 2'-O-Methyl RNA respectively). The inclusion of these synthetic blockers can restrict the activity of the exonucleases to those areas where they are required.

The incorporation of modified linkages can also modify the activities of restriction enzymes, resulting in double stranded cleavage, single stranded nicking or non-restriction of restriction enzyme recognition sites composed of the same base sequence. Deoxynucleotide phosphorothioates, methylated nucleotides and boronated deoxynucleoside triphosphates are all examples of suitable analogues which when incorporated into recognition sites could modify the activities of their restriction enzymes.

The use of chimeric RNA:DNA Amplification templates allows the selective removal of defined portions of the Template using RNase H which acts on the RNA portion of RNA:DNA hybrids.

According to a first embodiment of the present invention there is provided a method for detecting a target molecule, comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded amplification template comprising, arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence;

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence; and

iii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

b) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

c) a separating agent capable of removing sufficient of said extension nucleic acid sequence of said amplification template when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said amplification template to said complementary strand; and

d) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template;

iii) detecting any bound amplification template from the final amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

In the second embodiment, the present invention provides a method for detecting a target molecule (using amplification templates whose extension and hybridisation regions differ so as to minimise interfering side reactions) comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded first amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence;

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having a substantially different sequence to said extension nucleic acid sequence; and

iii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

b) a single stranded second amplification template comprising arranged in a 5' to 3' direction'

i) an extension nucleic acid sequence comprising said hybridisation nucleic acid sequence of said first amplification template;

ii) a hybridisation nucleic acid sequence comprising the extension nucleic acid sequence of said first amplification template; and

iii) an amplification moiety, being limited in all but the final amplification step to a nucleic acid sequence;

c) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no

previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said first and second amplification templates;

d) a separating agent capable of removing sufficient of said extension nucleic acid sequence of said first and second amplification templates when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said first and second amplification templates to said complementary strand; and

e) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequences of said first and second amplification templates;

iii) detecting any bound first and/or second amplification template from the final amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

If in the first and second embodiment the separation agent is a 5' double strand specific exonuclease, the hybridisation region of the amplification templates and the 5' terminus of the Locator probe would be protected by the inclusion of modified linkages or synthetic nucleotide analogues. If the separation agent is RNase H then the extension region of the amplification templates would be composed at least in part of RNA whilst the hybridisation region would be composed of DNA. Where the separating agents have temperature optima of 37°C, they could be used in conjunction with a polymerase such as Klenow (exo$^-$) polymerase. The method could then be envisioned to operate isothermally at 37°C.

If the separating agent used is a restriction endonuclease there is the potential to operate the system using a variety of formats as outlined below:

1. There is the potential to operate isothermally to provide a rapid qualitative result, or to temperature cycle to provide a less rapid but quantitative result.

2. The system could be operated at temperatures higher than 37°C to provide temperature controlled stringency, (an important facility given the length and complexity of the Amplification templates used).

3. The activities of restriction endonucleases are defined, well characterised and sequence based, more so than exonucleases such as T7 gene exonuclease and Lambda exonuclease, whose substrate specificity can vary.

4. The combination and concerted action of restriction endonucleases and polymerases under a single set of reaction conditions has been demonstrated frequently.

5. The latitude in operating temperatures allows for a more flexible approach in design rationale, particularly with respect to using different materials such as PNA and 2'- O-Methyl RNA.

This embodiment works by incorporating one or more restriction sites in the extension and hybridisation regions of the amplification templates. Those sites in the hybridisation region are protected by the incorporation of nucleotide analogues at the site of enzymatic cleavage. Those sites in the extension region are unmodified. Hybridisation of the amplification template and creation of a complement to the extension region results in the formation of double stranded recognition sites for the restriction enzymes. Use of one or more modified dNTPs in the reaction mix results in the newly synthesised strand being protected. The enzyme nicks the sites in the extension region of the amplification template and at the operating temperature used, or by elevating the temperature, the resultant fragments are dissociated. This provides a single stranded site to which an additional amplification template can hybridise to continue the assembly process.

It will be apparent to those practiced in the art that the restriction enzyme used in the description above must belong to that group which is capable of nicking the unprotected strand of a hemi-modified enzyme recognition site. It is also important that the recognition site sequence is non-palindromic such that amplification templates which self hybridise are not substrates for double stranded cleavage by the restriction enzyme. An example of a suitable enzyme would be *Bso*B1. It is also possible to use enzymes whose activity is naturally restricted to nicking of a single strand as opposed to double stranded cleavage. An example

of this kind of enzyme is N.*Bst*NB1 which has the recognition sequence 5'-GAGTCNNNNN-3'. When such an enzyme is used, it is unnecessary to include dNTP analogues in the reaction mix to protect the newly synthesised strand.

If the process operates isothermally the reaction should be rapid but qualitative in the signal output generated. Thermocycling (whereby nicked fragments dissociated at a higher temperature than the operating temperature of the restriction enzyme) could generate a signal output that provided quantitative information.

In the third embodiment, the present invention provides a method for detecting a target molecule, utilising a restriction enzyme and polymerase, and comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex, said amplification nucleic acid sequence having one or more restriction sites for a restriction endonuclease when hybridised to a complementary strand, but which by virtue of their sequence or due to nucleotide modifications are resistant to nicking by said restriction endonuclease;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence;

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence, and having nucleotide modifications which prevent its nicking by said restriction endonuclease; and

iii) an amplification moiety, being limited in all but the final amplification step to a nucleic acid sequence;

b) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no

previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

c) said restriction endonuclease; and

d) the reagents and conditions necessary to:

i) effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template, the reagents when required, including at least one modified nucleotide which, when incorporated into said complementary strand by said polymerising agent, prevents nicking of said complementary strand by said restriction endonuclease; and

ii) effect dissociation of nucleic acid strands which have been cut by said restriction endonuclease activity from uncut complementary strands whilst not effecting dissociation of uncut nucleic acid strands from uncut complementary strands;

iii) detecting any bound amplification template from the last amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

Amplification templates may consist of fewer than the three regions. A fourth embodiment of the present invention uses amplification templates that comprise in the 5-3' direction an extension nucleic acid region and a hybridisation nucleic acid region. The steps of hybridisation, extension and separation can be applied as with the first and second embodiments to generate a Primary Structure. This structure will consist of multiple repeats of the amplification template hybridisation region hybridised to the extended amplification moiety of the Locator probe. It will contain no amplification template amplification moieties. As with the first embodiment, a single amplification template whose extension and hybridisation region sequences are substantially the same can be used, or as with the second embodiment two amplification templates whose extension and hybridisation region sequences are substantially different can be employed.

Detection of this structure requires a denaturation and removal of the hybridised amplification templates prior to contacting the structure with a Detection probe

complementary to the extended Locator probe amplification moiety. If generation of a Secondary Structure rather than detection is required, after denaturation the structure is instead contacted with an additional amplification template comprising in the 5'-3' direction a hybridisation nucleic acid region and an amplification moiety. Any of this additional template which remains unhybridised can be removed by washing prior to addition of secondary amplification templates and generation of the Secondary Structure.

An advantage of this type of approach is that elevated temperature can be used as the separating agent. This additionally requires that the Locator probe be irreversibly linked to the target sequence to which it has hybridised (using for example a cross-linking agent such as DZQ [diazirinidylbenzoquinone]). Similarly if a Secondary Structure is to be generated it requires that the Hybridisation region of the amplification templates incorporated into the Primary Structure are cross-linked to the extended amplification moiety of the Locator probe.

In the fourth embodiment, the present invention provides a method for detecting a target molecule comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded primary amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence; and

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence;

b) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no

previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

c) a separating agent capable of removing sufficient of said extension nucleic acid sequence of said amplification template when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said amplification template to said complementary strand;

d) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template; and

e) a single stranded secondary amplification template comprising:

i) a hybridisation nucleic acid sequence comprising said primary amplification template hybridisation nucleic acid sequence; and

ii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

iii) detecting any bound secondary amplification template from the last amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

It is also possible to carry out the amplification process using amplification templates comprising in a 5'-3' direction a hybridisation and extension region, and whose sequences differ from each other. In the fifth embodiment, the present invention provides a method for detecting a target molecule, utilising amplification templates whose hybridisation and extension sequences differ, and comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded first primary amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence; and

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence;

b) a single stranded second primary amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence comprising said first primary amplification template hybridisation nucleic acid sequence; and

ii) a hybridisation nucleic acid sequence comprising said first primary amplification template extension nucleic acid sequence;

c) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

d) a separating agent capable of removing sufficient of said extension nucleic aid sequence of said amplification template when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said amplification template to said complementary strand;

e) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template;

f) a first single stranded secondary amplification template comprising:

i) a hybridisation nucleic acid sequence comprising said first primary amplification template hybridisation nucleic acid sequence; and

ii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence; and

g) a second single stranded secondary amplification template comprising:

i) a hybridisation nucleic acid sequence comprising said second primary amplification template hybridisation nucleic acid sequence; and

ii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

iii) detecting any bound secondary amplification template from the last amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

The invention will be further apparent from the following description, with reference to the several figures of the accompanying drawings, which show, by way of example only, forms of detection of target molecules and signal amplification.

Of the Figures:

Figure 1: shows forms of Locator probes hybridised to target nucleic acid sequences;

Figure 2: shows an amplification template used in the first aspect of the present invention;

Figure 3: shows a target molecule detection method according to the first aspect of the present invention;

Figure 4: shows incorrect amplification template binding;

Figure 5: shows amplification templates used in the second aspect of the present invention;

Figure 6: shows a target molecule detection method according to the second aspect of the present invention;

Figure 7: shows an amplification template used in the method of the third aspect of the present invention;

Figure 8: shows the method of the third aspect of the present invention; and

Figure 9: shows the method of the fourth aspect of the present invention.

As can be seen from Figure 1a, Locator probe 10 comprises binding nucleic acid moiety 11 joined by a 5'-5' linkage to amplification moiety 12. Binding nucleic acid moiety 11 hybridises target nucleic acid sequence 21 of target molecule 20. Figure 1b shows Locator probe 30 comprising binding moiety 31 which is rendered resistant to exonuclease activity (not shown), joined by a 5'-3' linkage to amplification moiety 32. Binding nucleic acid moiety 31 hybridises target nucleic acid sequence 21 of target molecule 20.

Figure 2 shows primary amplification template 40 comprising arranged in a 5'-3' direction extension region 41, exonuclease-resistant hybridisation region 42 and amplification moiety 43.

In the method of the first aspect of the present invention (Figure 3), hybridisation region 42 of amplification template 40 hybridises to amplification moiety 32 of Locator probe 30 (Figure 3a). DNA polymerase activity then causes the extension of amplification moiety 32, using extension region 40 as a template strand (Figure 3b). A 5' double-strand specific exonuclease (not shown) then digests the 5' terminus of the hybridised extension region 41. Exonuclease activity is halted when the enzyme encounters exonuclease resistant hybridisation region 42 (Figure 3c). Additional amplification template 40 is then able to hybridise to extended nucleic acid signal sequence 32 (Figure 3d) and the process of extension and exonuclease digestion proceed again to give the arrangement of Figure 3e.

Having washed the reaction mixture to remove unhybridised amplification template 40, the process is then repeated using secondary amplification template 50 comprising extension region 51, hybridisation region 52 and amplification moiety 53 (Figure 3f). Hybridisation region 52 is complementary to amplification moiety 43. The steps of extension of amplification moiety 43, digestion of extension region 51 and hybridisation of additional amplification template 50 then proceed as above, to create the Secondary Structure. This will incorporate large numbers of amplification moiety 53 which are available for hybridisation (Figures 3g-3j).

Detection probe 60 comprises signal detection nucleic acid sequence 61 complementary to amplification moiety 53, linked to label molecule 62. After hybridisation of Detection probe 60 and washing to remove any unhybridised probe, label molecules 62

are detected using standard techniques. The result of the detection step is then correlated with the presence of target sequence 21.

As can be seen from Figure 4, there are certain undesirable interactions that are possible between for example amplification moiety 32 of Locator probe 30 and extension region 41 of amplification template 40. Figure 4a shows extension region 41 hybridising to amplification moiety 32. This is then subject to exonuclease activity (Figure 4b) and, subsequently, hybridisation region 42 of amplification template 40 is able to hybridise to amplification moiety 32. However, since amplification template 40 no longer possesses an extension region 41, it is not possible to extend signal nucleic acid sequence 32 and no further Primary Structure assembly is possible.

Figure 5 shows pairs of amplification templates for use in the method of the second aspect of the present invention. Primary amplification template 70 comprises arranged in a 5' to 3' direction extension region 71, hybridisation region 72 and amplification moiety 73. Second primary amplification template 80 comprises arranged in a 5' to 3' direction extension region 81 (which has the same sequence as hybridisation region 72), hybridisation region 82 (which has the same sequence as extension region 71) and amplification moiety 73. Extension region 71 and hybridisation region 72 have substantially different sequences, such that they will not hybridise the same nucleic acid sequence. The first secondary amplification template 90 comprises arranged in a 5' to 3' direction extension region 91, hybridisation region 92 (which is complementary to amplification moiety 73 from the previous repeat) and amplification moiety 93. The second secondary amplification template 100 comprises arranged in a 5' to 3' direction extension region 101 (which has the same sequence as hybridisation region 92), hybridisation region 102 (which has the same sequence as extension region 91) and amplification moiety 103. Extension region 91 and hybridisation region 92 have substantially different sequences, such that they will not hybridise the same nucleic acid sequence. Nucleic acid sequences 72, 82, 92 and 102 contain substituted nucleic acid sequences, ensuring that they are resistant to 5' exonuclease activity.

In practice (Figure 6), target nucleic acid sequence 21 of target molecule 20 is hybridised by binding nucleic acid sequence 111 of Locator probe 110. Binding nucleic acid moiety 111 contains substituted nucleic acids that ensure that it is resistant to 5' exonuclease activity (Figure 6a). Hybridisation region 72 of first amplification template 70 then hybridises

to amplification moiety 112 of Locator probe 110 (Figure 6b). Since extension region 71 is different to hybridisation region 72 it is unable to hybridise to amplification moiety 112.

DNA polymerase activity then extends the free 3'-OH end of amplification moiety 112 using extension region 71 as a template. 5' exonuclease activity then digests extension region 71 (Figure 6c).

At this point it is possible for extension region 71 of amplification template 70 to hybridise to extended amplification moiety 112 (Figure 6d, top). However, since extension region 71 is not protected from 5' exonuclease activity, it will be digested, leaving extended amplification moiety 112 exposed (Figure 6e.1). The remains of partially digested amplification template 70 (comprising hybridisation region 72 and amplification moiety 73) are unable to hybridise to extended amplification moiety 112.

Alternatively (Figure 6d, bottom) hybridisation region 82 of amplification template 80 is able to hybridise to extended amplification moiety 112. Amplification moiety 112 is then further extended by DNA polymerase activity using extension region 81 as a template. Extension region 81 is then digested (Figure 6e.2) and assembly of the Primary Structure continues, with hybridisation region 72 of amplification template 70 hybridising to the extended amplification moiety 112.

After washing to remove unhybridised amplification templates 70 and 80 Secondary Structure assembly may then be performed using amplification templates 90 and 100. The Secondary Structure may then be detected and the results of the detection correlated with the presence of the target molecule. Figure 7 shows an amplification template used in an embodiment of the third aspect of the present invention. Amplification template 120 comprises extension region 121, hybridisation region 122 having substantially the same nucleic acid sequence as extension region 121 (at least to the extent that they are both able to hybridise the same nucleic acid sequence), and amplification moiety 123. Extension region 121 and hybridisation region 122 each in this example having two restriction sites 130, the restriction sites of extension region 121 being such that when hybridised to a complementary sequence they are capable of being cleaved by restriction endonuclease activity. However, the restriction sites of hybridisation region 122 have modified nucleotides such that they are not subject to restriction endonuclease activity.

Signal amplification using the method of the third aspect of the present invention is achieved as shown in Figure 8. Binding nucleic acid sequence 141 of Locator probe 140 hybridises to target nucleic acid sequence 21 of target molecule 20. The Locator probe amplification moiety 142 is complementary to hybridisation region 122 of amplification template 120 but has nucleotide substitutions such that it is not subject to restriction endonuclease activity. Hybridisation region 122 of amplification template 120 then hybridises to amplification moiety 142 (Figure 8a), which is then extended by DNA polymerase activity using extension region 121 as a template (Figure 8b). At least one of the solution phase dNTPs utilised by the polymerase is modified such that its incorporation renders the newly synthesised strand resistant to restriction endonuclease activity.

Restriction endonuclease activity then nicks extension region 121. In an isothermal assay format the resultant fragments would be so designed as to dissociate at the operating temperature used. In a thermocycled format the temperature would be elevated to allow dissociation of the fragments without concomitant dissociation of uncleaved sequences. Additional amplification template 120 is then able to hybridise to extended amplification moiety 142 and the reaction able to proceed to assemble the Primary Structure. Detection or Secondary Structure assembly can be subsequently carried out by repetition of the same basic processes as earlier described.

In the fourth embodiment of the present invention (Figure 9), target nucleic acid sequence 21 of target molecule 20 is hybridised to by Binding moiety 151 (which can have nucleotide modifications to render it resistant to 5' double stranded exonuclease activity should that be required) of Locator probe 150 (Figure 9a). Amplification moiety 152 is linked to binding nucleic acid sequence 151 by extension blocker 153 such that it has an exposed 3'-OH group. When amplification moiety 152 is acting as the template strand for the synthesis of a complementary strand, the extension blocker prevents the progress of any DNA polymerase beyond the end of the amplification moiety, thereby preventing the displacement of binding moiety 151 from target nucleic acid sequence 21. Once unbound Locator probe has been removed, binding moiety 151 may optionally be cross-linked to target sequence 21 prior to Primary Structure assembly.

In use, hybridisation region 162 of amplification template 160 hybridises to amplification moiety 152 (Figure 9b). Hybridisation region 162 has an exposed 3'-OH group, as does amplification moiety 152. Extension region 163 then acts as a template strand for the extension of amplification moiety 152, which in turn acts as a template strand for the extension of hybridisation region 161 as far as extension blocker 153 (Figure 9c). 5' double strand exonuclease activity could then digest extended amplification template 160 as shown in this example, leaving free extended amplification moiety 152 (Figure 9d). Alternatively the amplification templates could be completely removed through the use of elevated temperature. Additionally the amplification templates could be so designed as to contain modified and unmodified restriction sites that would allow endonuclease cleavage to remove the extension region of the amplification template only.

Additional amplification template 160 is then able to hybridise to extended amplification moiety 152 (Figure 9e) and extension region 162 again able to act as template strand for the synthesis of a complementary strand, extending further amplification moiety 152. Similarly, amplification moiety 152 is able act as template strand for the extension of hybridisation region 161 (Figure 9f). Removal of part or all of the extended amplification template 160 can then be achieved through 5' exonuclease activity (as in this example), or by elevated temperature, or by a restriction endonuclease (Figure 9g, 9h).

Once amplification moiety 152 has been sufficiently extended, it may be detected by hybridisation of Detection probes complementary to the repeats within the extended strand. Alternatively secondary amplification templates, comprised in the 5'-3' direction of hybridisation region 162, extension blocker 153, and amplification moiety 173 can be hybridised to extended amplification moiety 152 (Figures 9i, 9j). The hybridisation region may incorporate modified nucleotides (as in this example) to render it resistant to 5' double stranded exonuclease activity should that be required.

Amplification moiety 173 is then be used as the target for an additional round of signal amplification performed as before using amplification template 180 comprised in the 5'-3' direction of hybridisation region 181 and extension region 182 (Figures 9j-9p). A final signal detection step can then be performed (Figure 9q) which detects the extension region 181 of amplification template 180.

Examples 1 and 2 below detail specific reagents and conditions used to achieve target molecule detection and signal amplification using the methods of the present invention. It will be appreciated that it is not intended to limit the invention to the these examples only, many variations, such as might readily occur to one skilled in the art, being possible, without departing from the scope thereof as defined by the appended claims.

Example 1

Concerted action of T7 gene 6 exonuclease and Klenow (exo-) polymerase.

CMV-002, a 24-mer sequence specific for a conserved region in the *GlyB* gene of CMV, was covalently linked to solid phase support and used as the target for the amplification template SA-EX1. A Detection Oligonucleotide SA-B1 was designed to hybridise to the site generated by SA-EX1 in combination with the concerted action of Klenow (exo-) polymerase and T7 gene 6 exonuclease. The sequence and composition of these two oligonucleotides and the biotin labelled detection oligonucleotide SA-B1 are given below.

CMV-002: 5'- TCG ACG GTG GAG ATA CTG CTG AGG – 3'

SA-EX1: 5'- *TTC* TCC TTC CAG TTG CTA **CCU CAG CAG** TAT CTC CAC –3'

SA-B1: 5'- TTC TCC TTC CAG TTG CTA – 3'

Bases shown in italics contain phosphorothioate linkages. Bases shown in bold are 2'-O-Methyl RNA. Bases shown underlined have a biotin label attached. All oligonucleotides were supplied by Oswel (Southampton UK).

76 pmoles of SA-EX1 was added to 2mg of CMV-002 support in each of four 0.2 ml eppendorf tubes in a total volume of 40 µl of 1 x Klenow (exo-) buffer. Hybridisation was allowed to proceed at room temperature for 10 minutes with gentle mixing by inversion at 1 minute intervals.

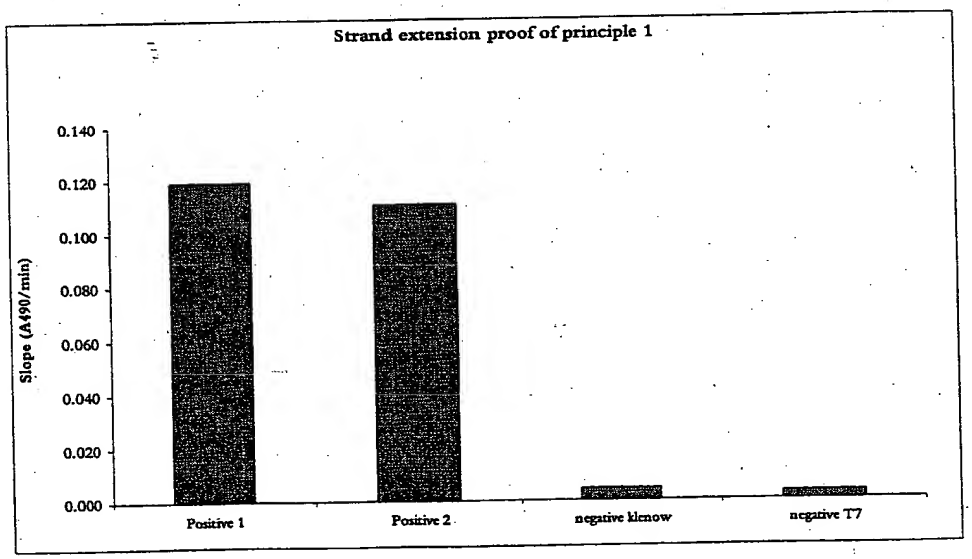
To tubes A and B, 10 µl of 1 x Klenow buffer containing 10 units of Klenow (exo-) polymerase, 100 units of T7 gene 6 exonuclease and 2 µl of a 20mM dNTP mix was added. To tube C, 10 µl of the same mixture omitting the polymerase was added, and to tube D, 10 µl of the mixture, omitting the T7 exonuclease was added. Samples were gently mixed and incubated at 37°C for 15 minutes.

Samples were transferred to bottom fritted DARAS™ columns. 100 µl of 1 x Sample Buffer (50mM Sodium citrate, 80mM Sodium chloride, 8 mM Magnesium chloride, 10mM Tris.HCl pH 8.3) was dispensed through each column in triplicate.

50 µl of 1 x Sample Buffer containing 79 pmoles of SA-B1 was added to each column and the column contents mixed by periodic inversion for 10 minutes at room temperature. Columns were washed by passage of 100 µl of System Buffer (10 mM Tris.HCl, pH 8.3) through each in triplicate.

The presence of hybridised SA-B1 oligonucleotide was confirmed using the EDSA1 detect protocol on the DARAS™ system. Results are presented in Fig. 1 as the slope of the signal detected (absorbance change at 490 nm /minute).

Fig 1



Example 2

Strand extension by sequential hybridisation..

CMV-002, a 24-mer sequence specific for a conserved region in the *GlyB* gene of CMV, was covalently linked to solid phase support and used as the target for the EDSA amplification template SA-EX1. A second amplification template, SA-EX22, was designed to hybridise to the site generated by SA-EX1 through the concerted action of Klenow (exo-) polymerase and T7 exonuclease. A Detection oligonucleotide SA-B2 was designed to hybridise to the site generated by SA-EX22 through the concerted action of Klenow (exo-) polymerase and T7 exonuclease. The sequence and composition of the Target (CMV-002), Amplification (SA-EX1, SA-EX22) and Detection (SA-B2) oligonucleotides are given below.

CMV-002: 5'- TCG ACG GTG GAG ATA CTG CTG AGG – 3'

SA-EX1: 5'- *TTC* TCC TTC CAG TTG CTA **CCU CAG CAG** TAT CTC CAC –3'

SA-EX22: 5'-*GTG* AAG ATG TTG CAT GTT CTC **CUU CCA** GTT GCT ACC- 3'

SA-B2: 5'- GTG AAG ATG TTG CAT GTT – 3'

Bases shown in italics contain phosphorothioate linkages. Bases shown in bold are 2'-O-Methyl RNA. Bases shown underlined have a biotin label attached. All oligos were supplied by Oswel (Southampton UK). All enzymes and buffers were supplied by Amersham Life Sciences Inc.(UK)

10 pmoles of SA-EX1 was added to 2mg of CMV-002 support in each of six 0.2 ml eppendorf tubes in a total volume of 30 µl of 1 x Klenow (exo-) buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT 50 µg/ml BSA). Hybridisation was allowed to proceed at room temperature for 10 minutes, with gentle mixing by inversion at one minute intervals. The solution phase was removed by aspiration, and 50 µl of 1 x Klenow Buffer added. After the beads settled, the upper phase was removed by aspiration. This was repeated two more times.

To tubes A - E, 30 µl of 1 x Klenow buffer containing 15 units of Klenow (exo-) polymerase, 100 units of T7 gene 6 exonuclease, 2 µl of a 20mM dNTP mix and 100 pmoles of SA-EX22 was added. To tube F, 30 µl of the same mixture omitting both the polymerase

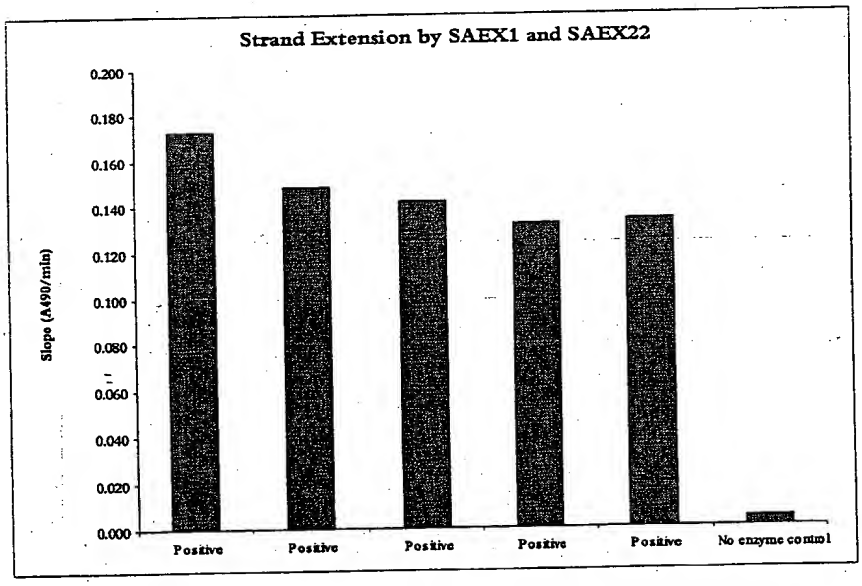
and the T7 exonuclease was added. Samples were gently mixed and incubated at 37°C for 30 minutes.

Samples were transferred to bottom fritted DARAS™ columns. 100 µl of 1 x Sample Buffer (50mM Sodium citrate, 80mM Sodium chloride, 8 mM Magnesium chloride, 10mM Tris.HCl pH 8.3) was dispensed through each column in triplicate.

50 µl of 1 x Sample Buffer containing 1 pmoles of SA-B2 was added to each column and the column contents mixed by periodic inversion for 10 minutes at room temperature. Columns were washed by passage of 100 µl of System Buffer (10 mM Tris.HCl, pH 8.3) through each in triplicate.

The presence of labelled SA-B2 oligonucleotide was confirmed using the EDSA1 detect protocol on the DARAS™ system. Results are presented in Fig. 1 as the slope of the signal detected (absorbance change at 490 nm /minute).

Fig 1



CLAIMS

1. A method of detecting a target molecule, comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence;

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence; and

iii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

b) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

c) a separating agent capable of removing sufficient of said extension nucleic acid sequence of said amplification template when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said amplification template to said complementary strand; and

d) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template;

iii) detecting any bound amplification template from the final amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

2. A method for detecting target molecule according to claim 1, the removal of said extension nucleic acid sequence being achieved by the use of a 5' double stranded exonuclease against whose activity the hybridisation nucleic acid sequence is protected.

3. A method for detecting a target molecule comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded first amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence;

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having a substantially different sequence to said extension nucleic acid sequence; and

iii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

b) a single stranded second amplification template comprising, arranged in a 5' to 3' direction',

i) an extension nucleic acid sequence comprising said hybridisation nucleic acid sequence of said first amplification template;

ii) a hybridisation nucleic acid sequence comprising the extension nucleic acid sequence of said first amplification template; and

iii) an amplification moiety, being limited in all but the final amplification step to a nucleic acid sequence;

c) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no

previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said first and second amplification templates;

d) a separating agent capable of removing sufficient of said extension nucleic acid sequence of said first and second amplification templates when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said first and second amplification templates to said complementary strand; and

e) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequences of said first and second amplification templates;

iii) detecting any bound first and/or second amplification template from the final amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

4. A method of detecting a target molecule according to claim 3, the removal of said extension nucleic acid sequence of said first and second amplification templates being achieved by the use of a 5' double-stranded exonuclease against whose activity said hybridisation nucleic acid sequence of said first amplification template and said hybridisation nucleic acid sequence of said second amplification template are protected.

5. A method for detecting a target molecule comprising the steps of:

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex, said amplification nucleic acid sequence having one or more restriction sites for a restriction endonuclease when hybridised to a complementary strand;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence;

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence; and

iii) an amplification moiety, being limited in all but the final amplification step to a nucleic acid sequence;

b) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

c) said restriction endonuclease; and

d) the reagents and conditions necessary to:

i) effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template; and

ii) effect dissociation of nucleic acid strands which have been cut by said restriction endonuclease activity from uncut complementary strands whilst not effecting dissociation of uncut nucleic acid strands from uncut complementary strands;

iii) detecting any bound amplification template from the last amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

6. A method according to claim 5, said amplification nucleic acid sequence and said hybridisation nucleic acid sequence having nucleotide modifications which prevent cleavage by said restriction endonuclease, and said reagents including at least one modified nucleotide which, when incorporated into said complementary strand by said polymerising agent, prevent cleavage of said complementary strand by said restriction endonuclease.

7. A method according to claim 5, said amplification nucleic acid sequence and said hybridisation nucleic acid sequence having nucleotide modifications which prevent cleavage by said restriction endonuclease, said restriction enzyme having single stranded nicking activity only.

8. A method according to claims 5-7, being performed isothermally.

9. A method according to claims 5-7, being performed at more than one temperature.

10. A method for detecting a target molecule comprising the steps of;

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded primary amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence; and

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence;

b) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

c) a separating agent capable of removing sufficient of said extension nucleic acid sequence of said amplification template when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said amplification template to said complementary strand;

d) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no

previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template; and

e) a single stranded secondary amplification template comprising:

i) a hybridisation nucleic acid sequence comprising said primary amplification template hybridisation nucleic acid sequence; and

ii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

iii) detecting any bound secondary amplification template from the last amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

11. A method for detecting a target molecule comprising the steps of;

i) contacting a sample with a locator probe comprising a binding moiety specific for said target molecule and an amplification nucleic acid sequence to produce a target molecule-locator probe complex;

ii) producing an amplification structure bound to any complex produced in step (i) by performing one or more times the amplification step of treating said sample and locator probe with:

a) a single stranded first primary amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence; and

ii) a hybridisation nucleic acid sequence complementary to the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) and having substantially the same sequence as said extension nucleic acid sequence;

b) a single stranded second primary amplification template comprising arranged in a 5' to 3' direction:

i) an extension nucleic acid sequence comprising said first primary amplification template hybridisation nucleic acid sequence; and

ii) a hybridisation nucleic acid sequence comprising said first primary amplification template extension nucleic acid sequence;

c) a polymerising agent capable of extending the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no

previous amplification step, of step (i) by synthesising a complementary strand to said extension nucleic acid sequence of said amplification template;

d) a separating agent capable of removing sufficient of said extension nucleic aid sequence of said amplification template when hybridised to said complementary strand to allow subsequent hybridisation of said hybridisation nucleic acid sequence of said amplification template to said complementary strand;

e) the reagents and conditions necessary to effect the action of said polymerising agent and separating agent to allow the extension of the 3' terminus of the amplification nucleic acid sequence of the previous amplification step or, where there is no previous amplification step, of step (i) by the synthesis of a plurality of sequences complementary to said extension nucleic acid sequence of said amplification template;

f) a first single stranded secondary amplification template comprising:

i) a hybridisation nucleic acid sequence comprising said first primary amplification template hybridisation nucleic acid sequence; and

ii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence; and

g) a second single stranded secondary amplification template comprising:

i) a hybridisation nucleic acid sequence comprising said second primary amplification template hybridisation nucleic acid sequence; and

ii) an amplification moiety, being limited in all but the final repeat to a nucleic acid sequence;

iii) detecting any bound secondary amplification template from the last amplification step; and

iv) correlating the results of detection step (iii) with the presence of said target molecule.

12. A method for detecting a target nucleic acid molecule according to either one of claims 10 or 11, the removal of said primary amplification template being achieved by the use of a 5' double strand specific exonuclease.

13. A method for detecting a target nucleic acid molecule according to either one of claims 10 or 11, the removal of said primary amplification template being achieved through the use of elevated temperature.

14. A method for detecting a target molecule according to any one of claims 1-9, the step of detecting any bound amplification template comprising the steps of:

i) treating said sample, locator probe and amplification template or amplification templates with a detection probe which binds specifically to said amplification moiety of the last of said amplification templates; and

ii) detecting any bound detection probe.

15. A method for detecting a target molecule according to any one of claims 10-13, the step of detecting any bound amplification template comprising the steps of:

i) treating said sample, locator probe, primary amplification template and secondary amplification template with a detection probe which binds specifically to said amplification moiety of the last of said secondary amplification templates; and

ii) detecting any bound detection probe.

16. A method according to either one of claims 14 or 15, the detection probe having a label which is detected by any one of the group of luminometry, fluorometry, spectrophotometry, and radiometry.

17. A method according to claim 16, the detection probe being labelled with any one of the group of, FAM (carboxyfluorescein), HEX (hexachlorofluorescein), TET (tetrachlorofluorescein), ROX (carboxy-X-rhodamine), TAMRA (carboxytetramethylrhodamine), JOE (carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), or with biotin.

18. A method according to any one of the preceding claims, the amplification step being performed two or more times, each amplification step being performed using an amplification template having a different extension nucleic acid sequence, hybridisation nucleic acid sequence and amplification moiety to that of the amplification template used in the previous amplification step.

19. A method according to any one of the preceding claims, the target molecule to be detected being a nucleic acid sequence and the binding moiety of said locator probe comprising a nucleic acid sequence complementary to said target molecule nucleic acid sequence.

20. A method according to any one of the preceding claims, being performed using more than one locator probe, each locator probe having the same amplification nucleic acid sequence.

21. A method according to any one of the preceding claims, comprising two repeats.

22. A method according to any one of the preceding claims, unreacted reagents being removed at the end of step (i), each repeat, or detection step by washing.

23. A method according to claim 22, the unreacted reagents being selected from the group of locator probe, amplification template, primary amplification template, secondary amplification template and detection probe.

Fig. 1

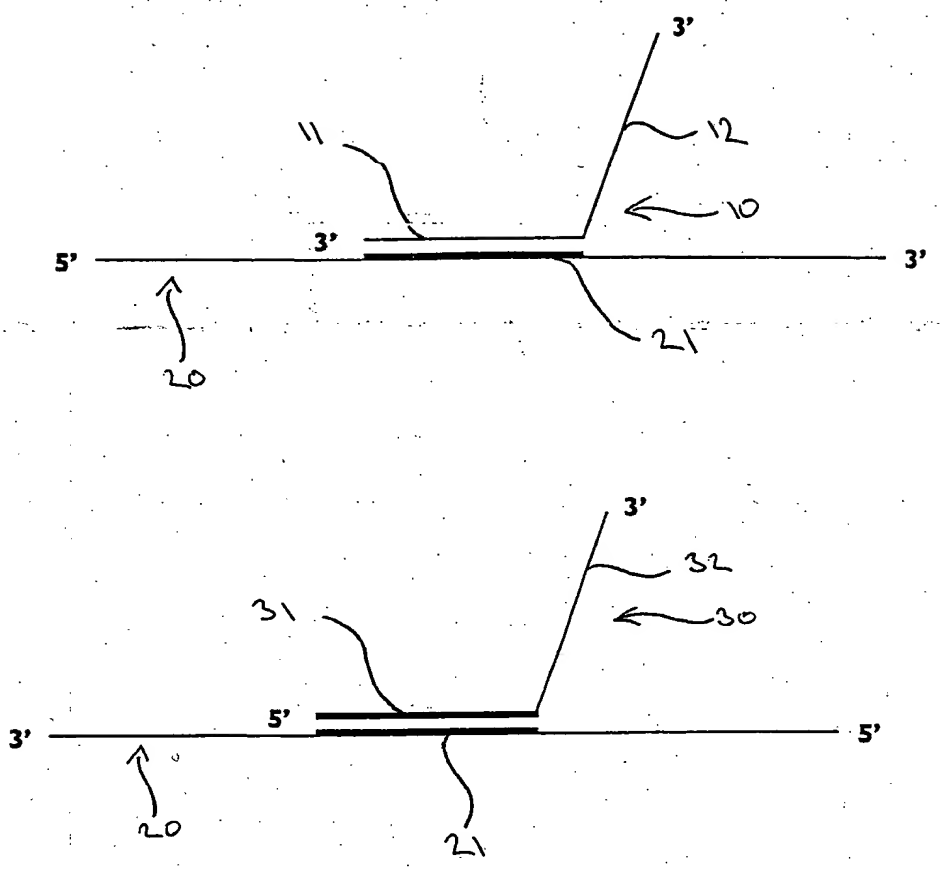


Fig. 2

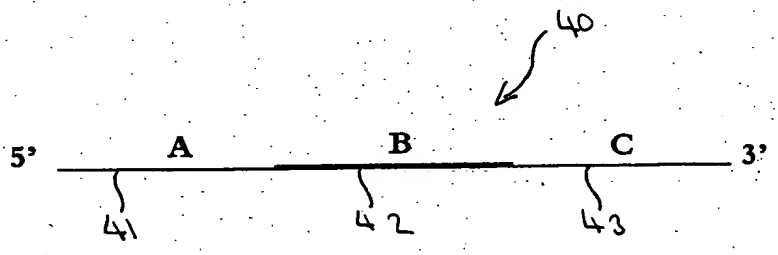
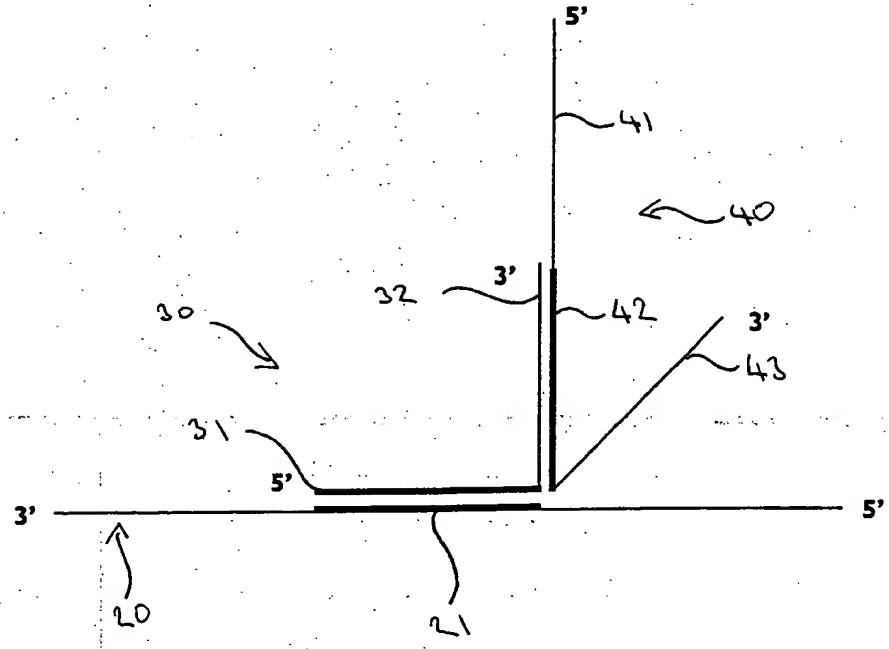


Fig. 3

a)



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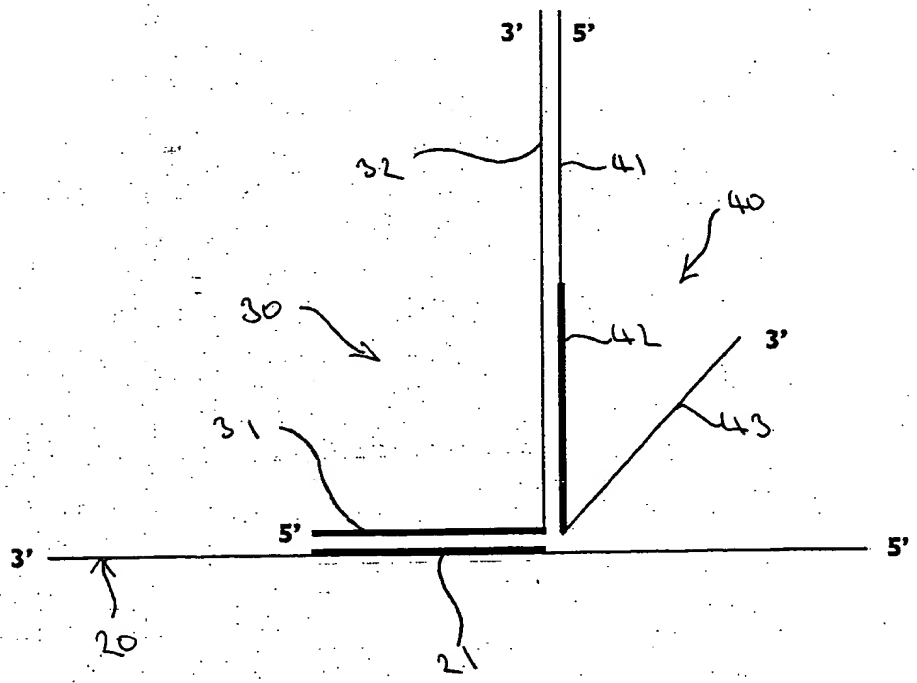
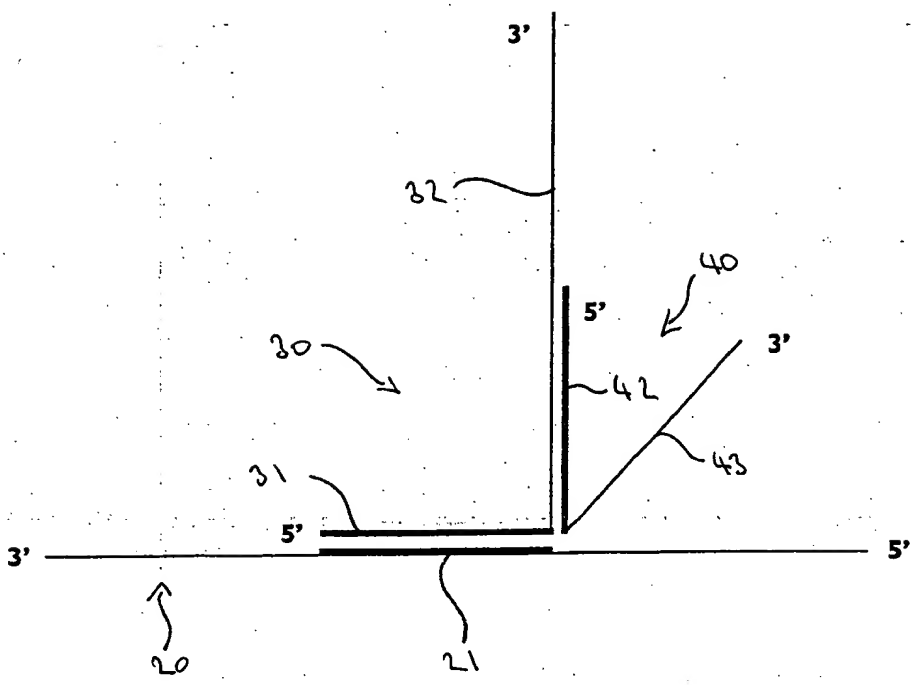


c)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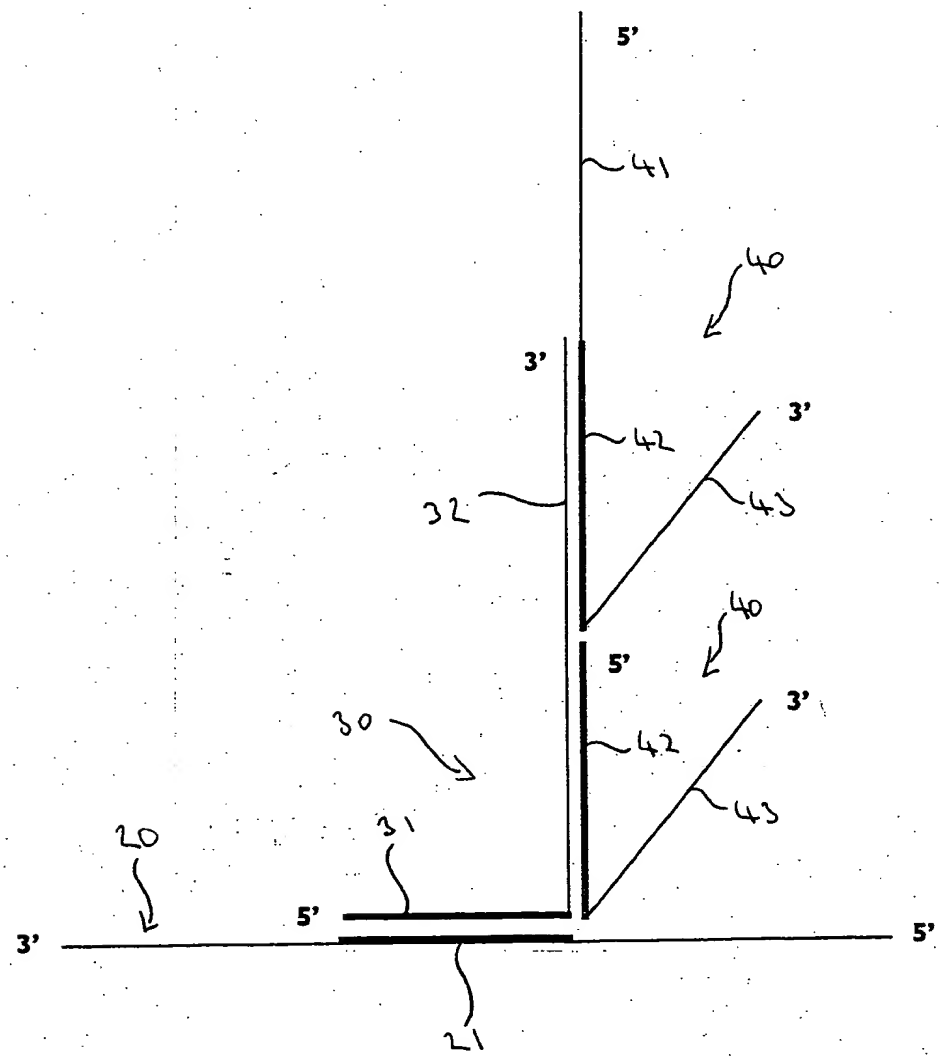
d)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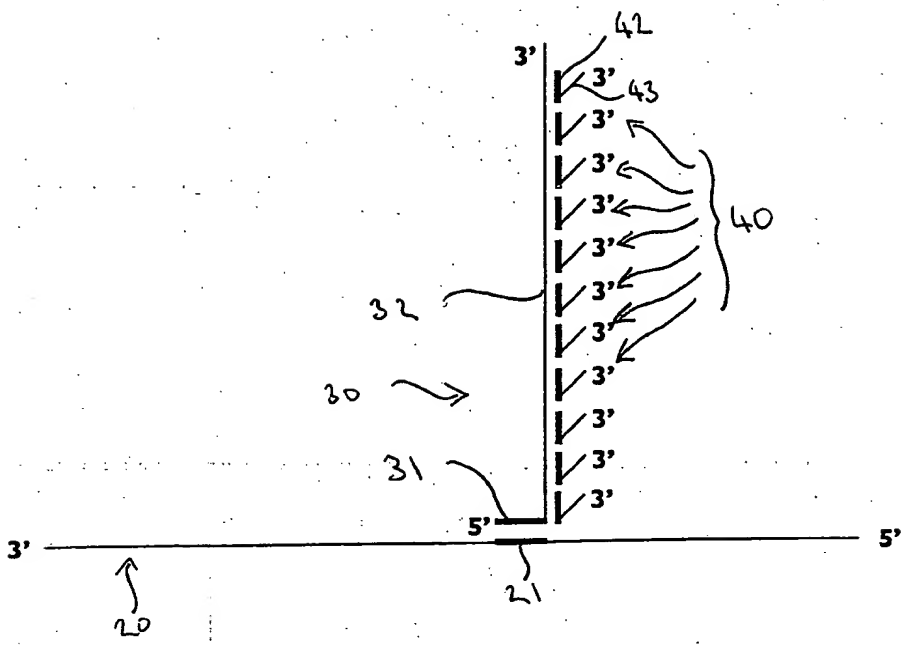
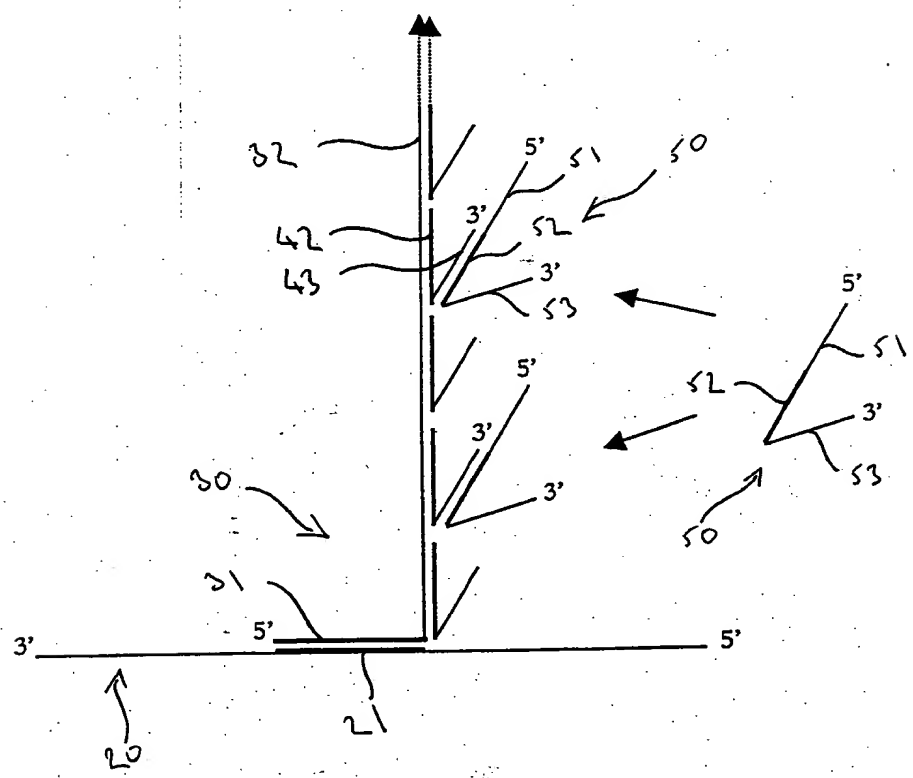
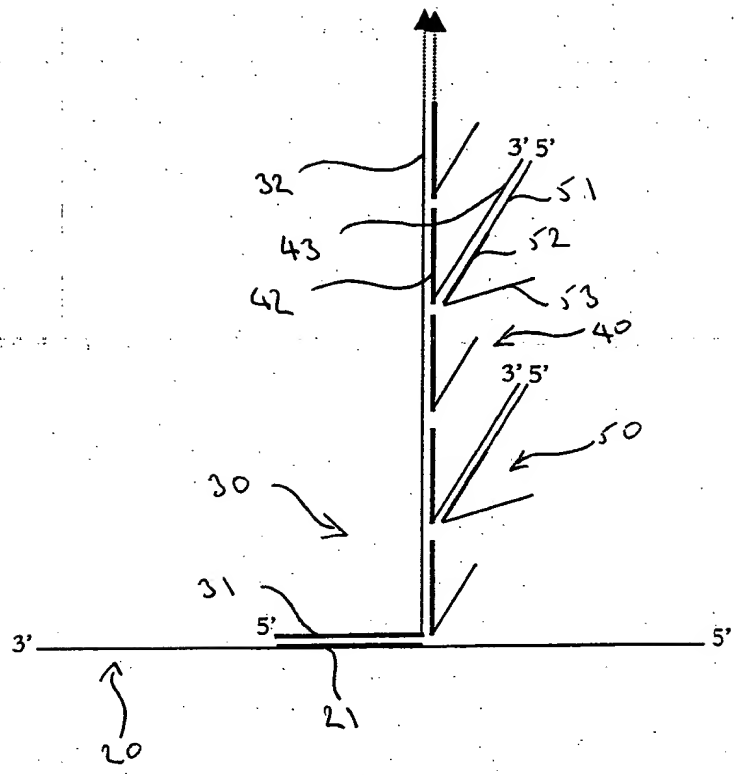
e)



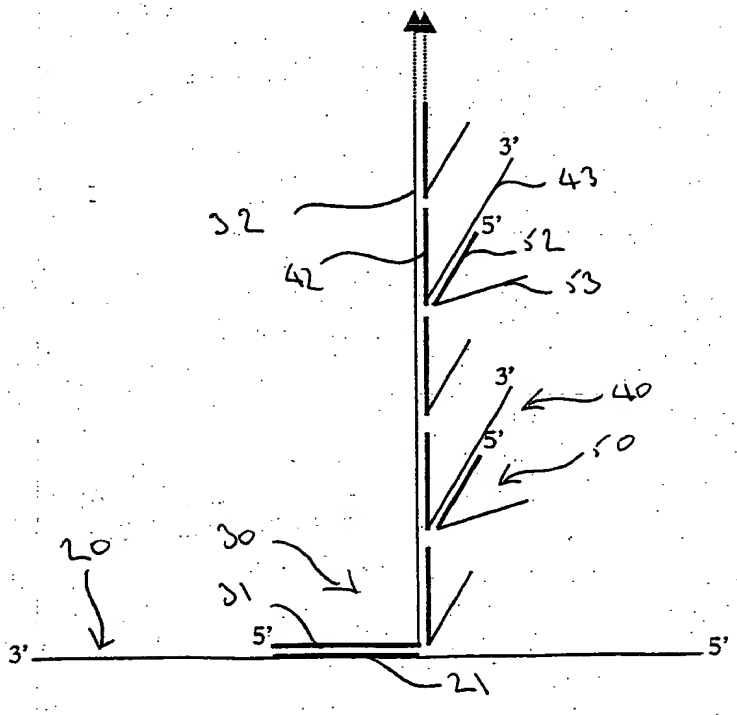
f)



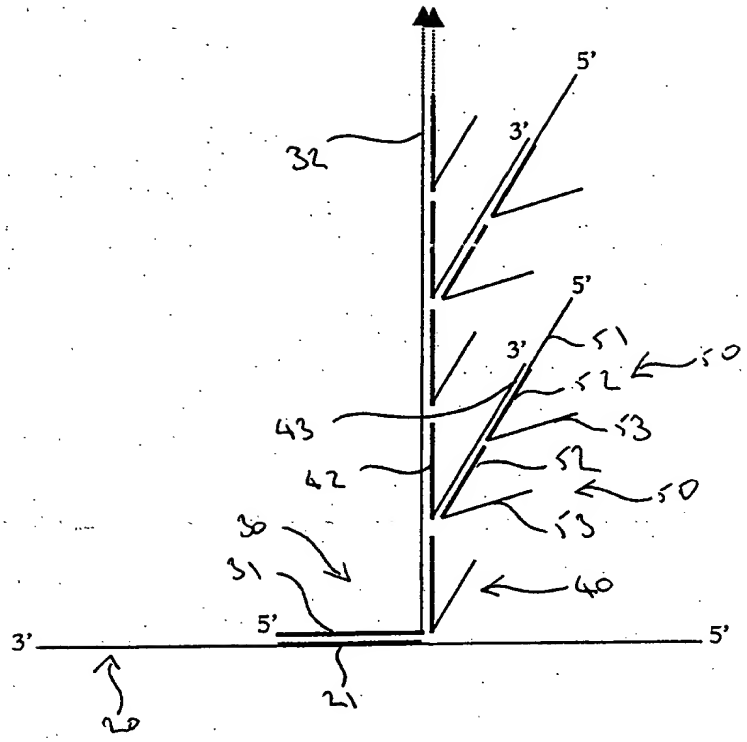
g)


3' 5'
32
51
43
52
42
53
40
3' 5'
50
30
31
5'
3'
5'
21
20

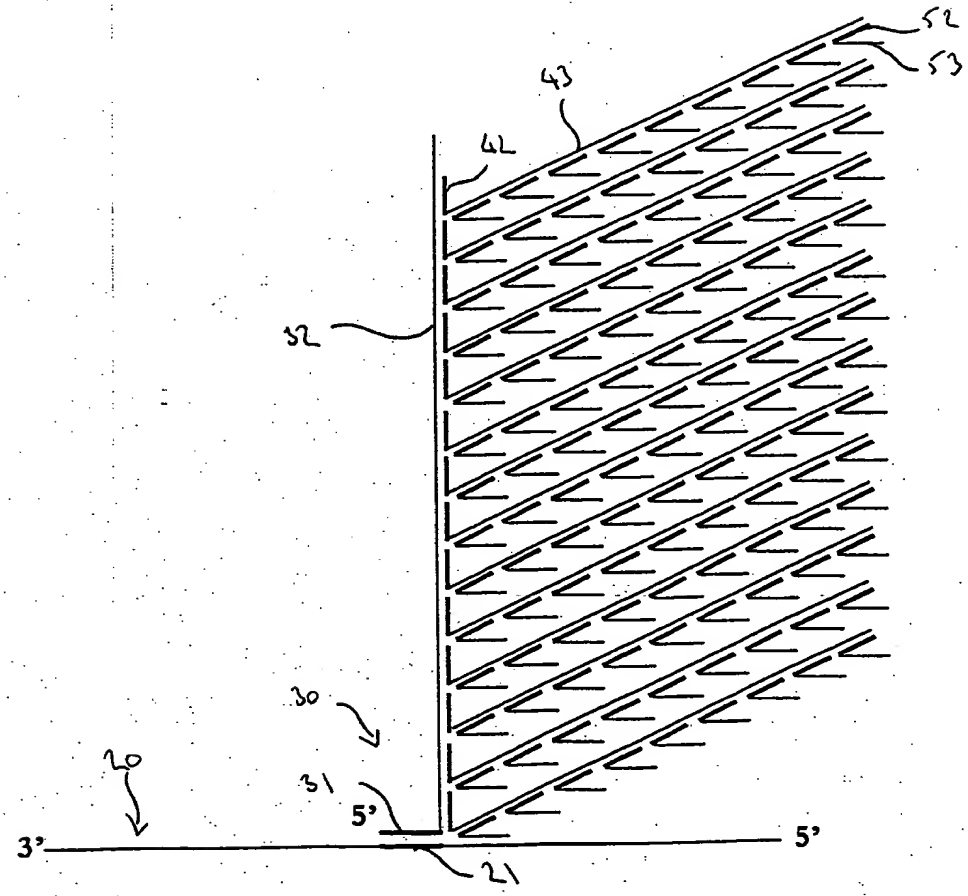
h)


3'
43
32
5'
52
42
53
3'
40
5'
50
20
30
31
5'
3'
5'
21

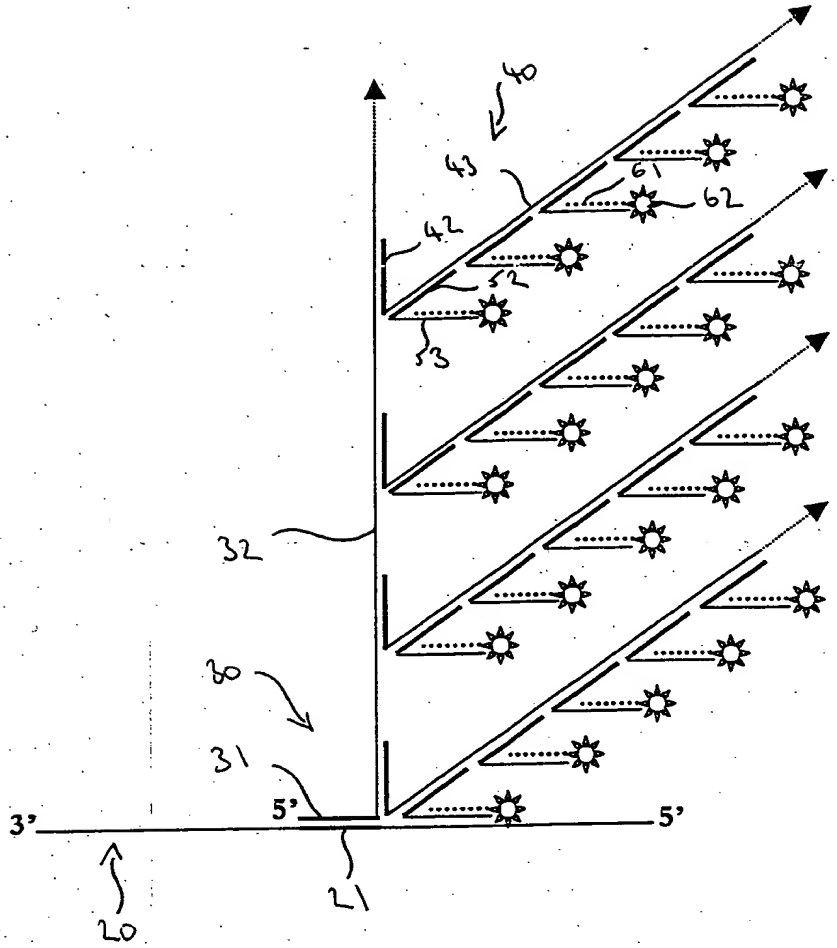
i)


5'
3'
32
5'
3'
51
50
52
43
53
42
52
50
53
30
31
40
5'
3'
5'
20
21

j)


52
53
43
42
52
30
20
31
5'
3'
5'
21

k)



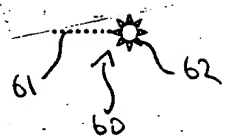
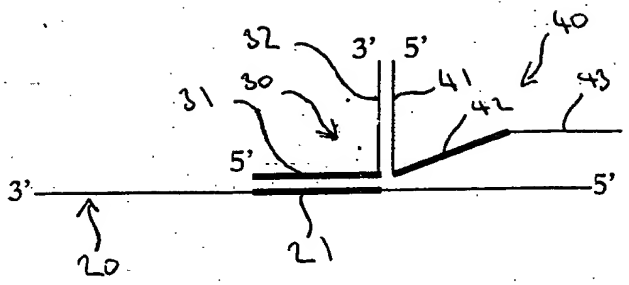
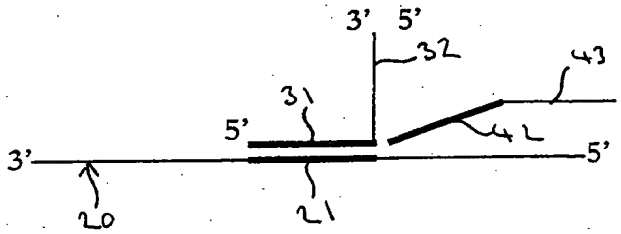


Fig. 4

a)



b)



c)

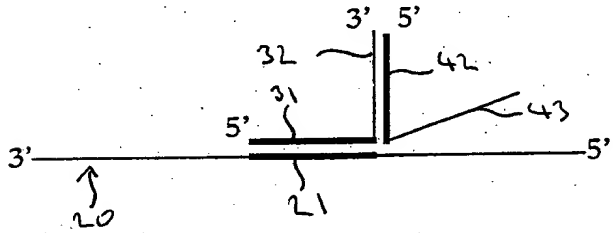


Fig. 5

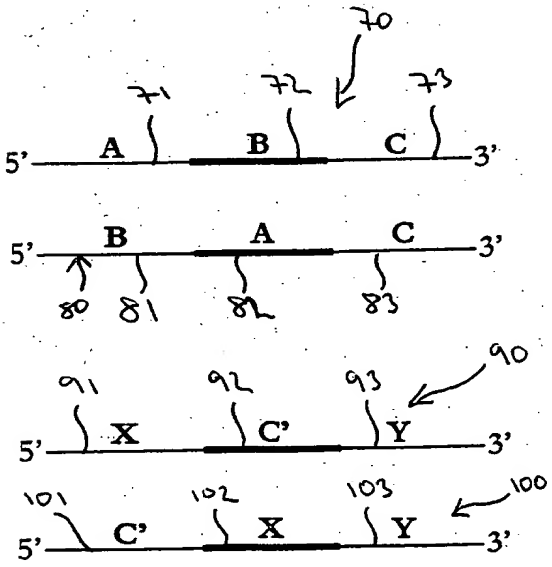
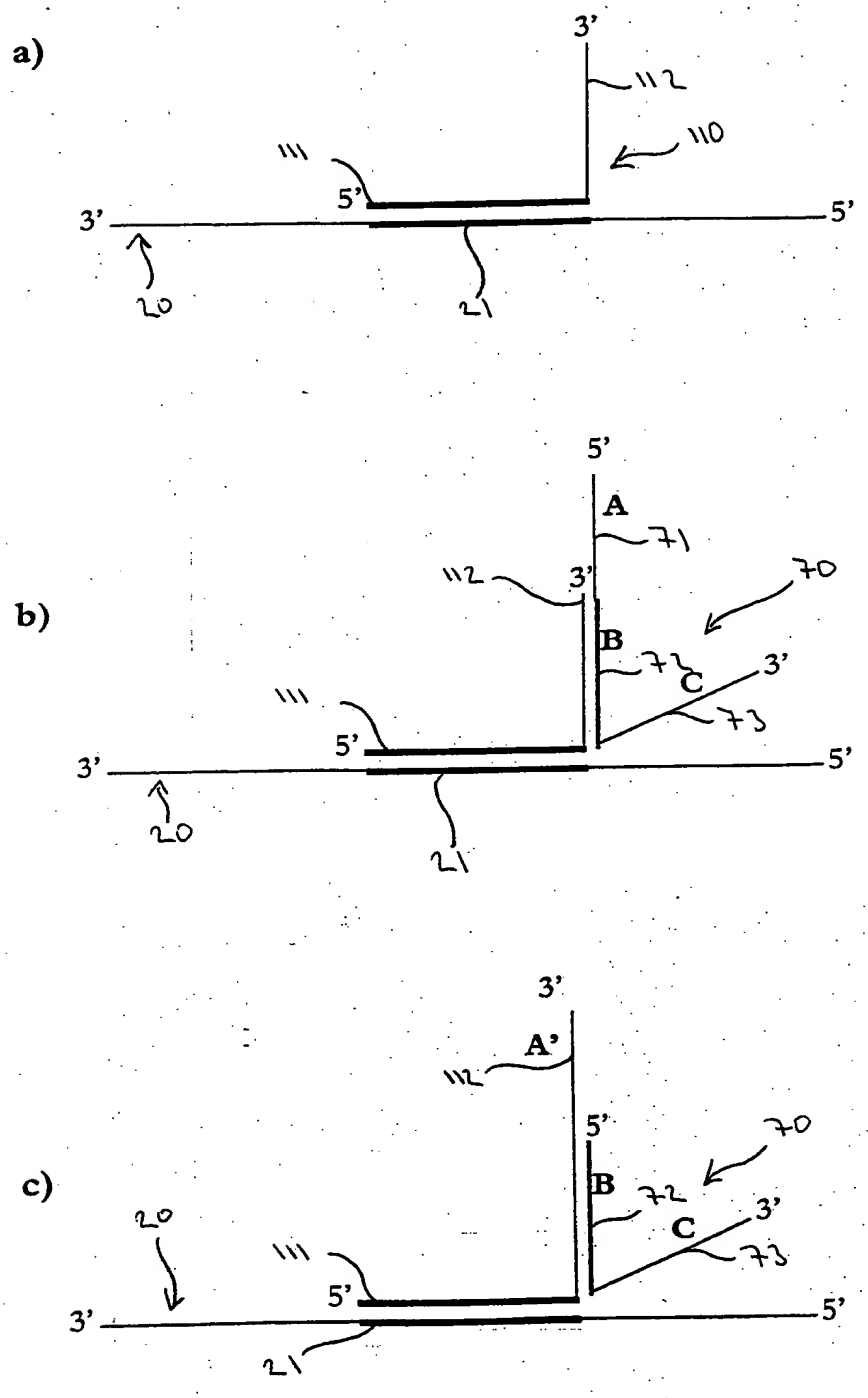
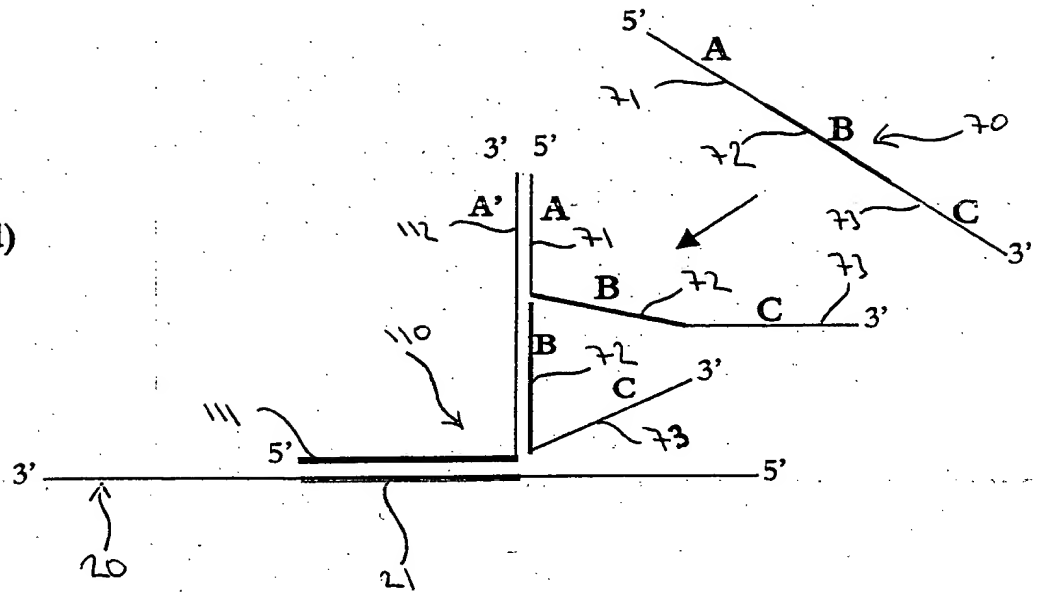
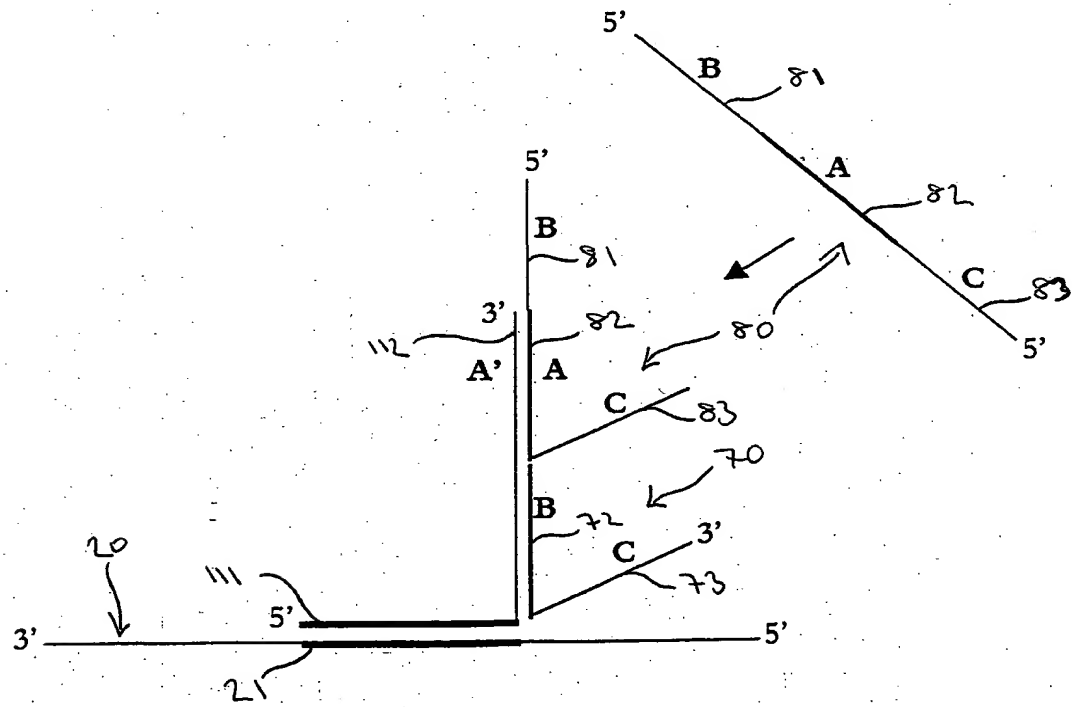


Fig. 6

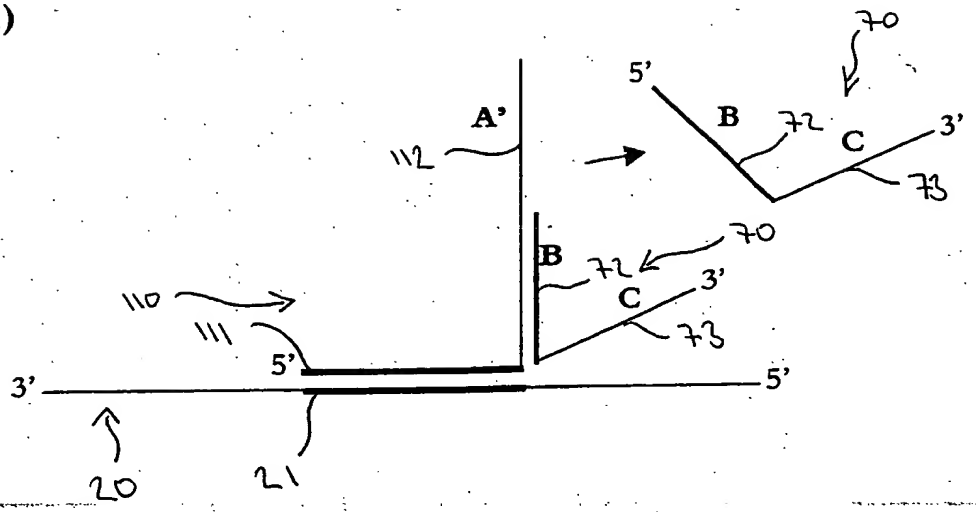

a)
3'
112
110
111
5'
3'
5'
20
21
b)
5'
A
71
112
3'
70
B
72
C
3'
73
111
5'
3'
5'
20
21
c)
3'
A'
112
5'
70
B
72
C
3'
73
20
111
5'
3'
5'
21

d)


5'
A
71
B
70
72
C
73
3'
3' 5'
A'
A
112
71
B
72
C
73
3'
110
B
72
C
3'
73
111
5'
3'
5'
20
21


5'
B
81
A
82
C
83
5'
5'
B
81
3'
82
112
A'
A
80
C
83
70
B
72
C
3'
73
20
111
5'
3'
5'
21

e.1)


70
5'
B
72
C
3'
73
A'
112
B
72
70
C
3'
73
110
111
5'
3'
5'
20
21

e.2)

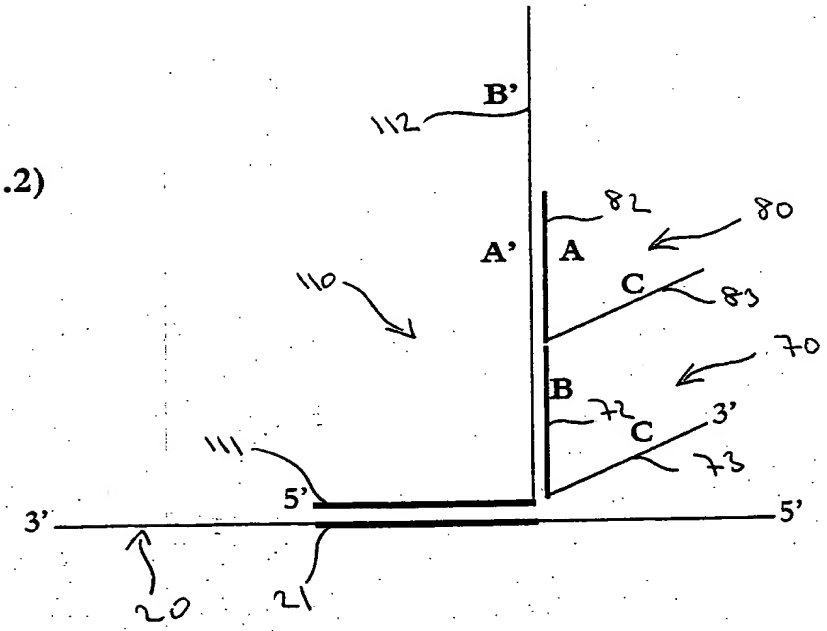

B'
112
82
80
A'
A
C
83
110
70
B
72
C
3'
73
111
5'
3'
5'
20
21

Fig. 7

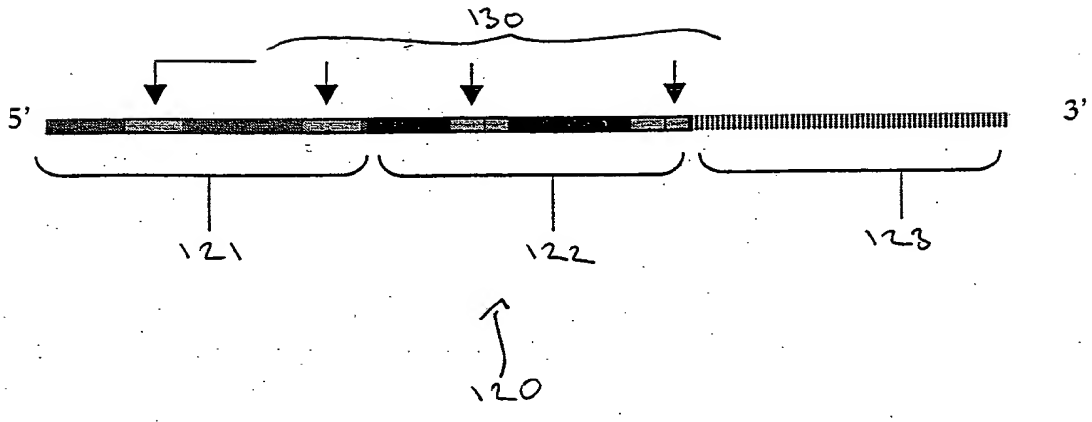
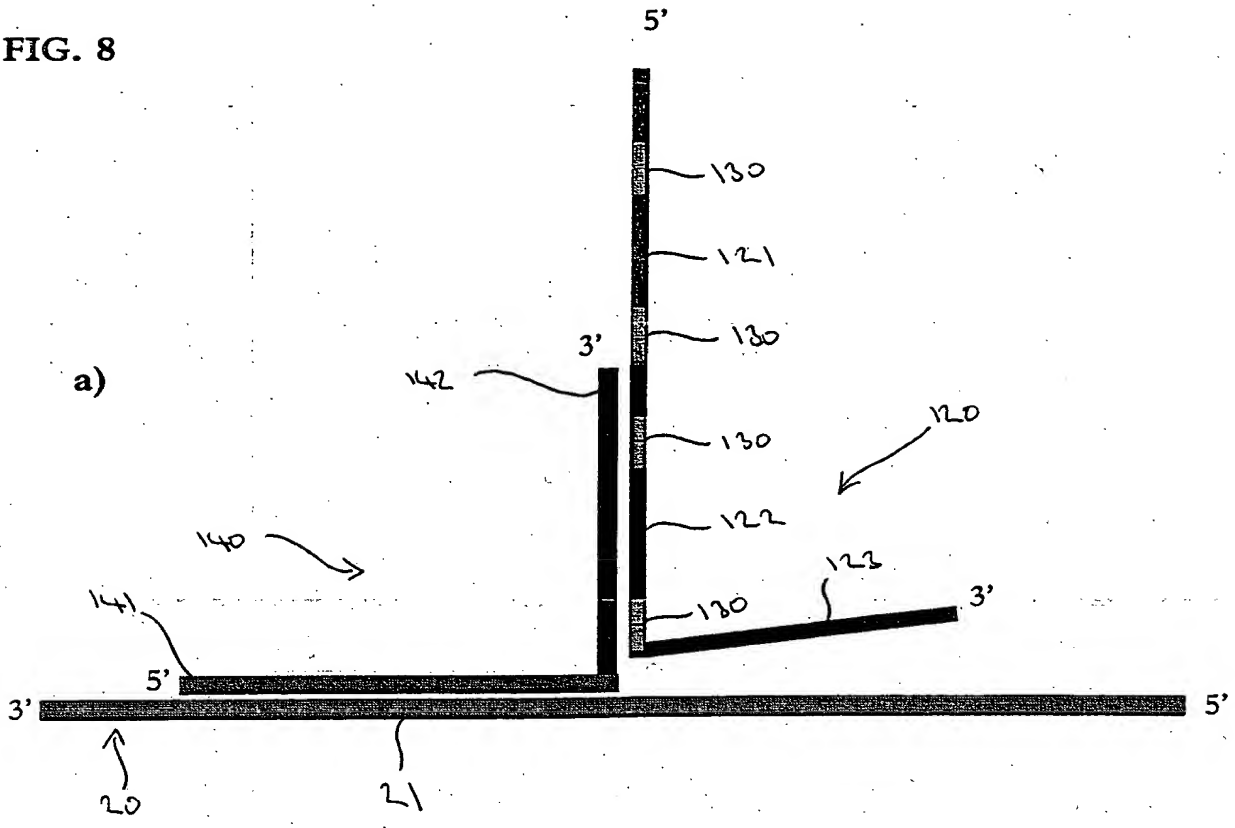
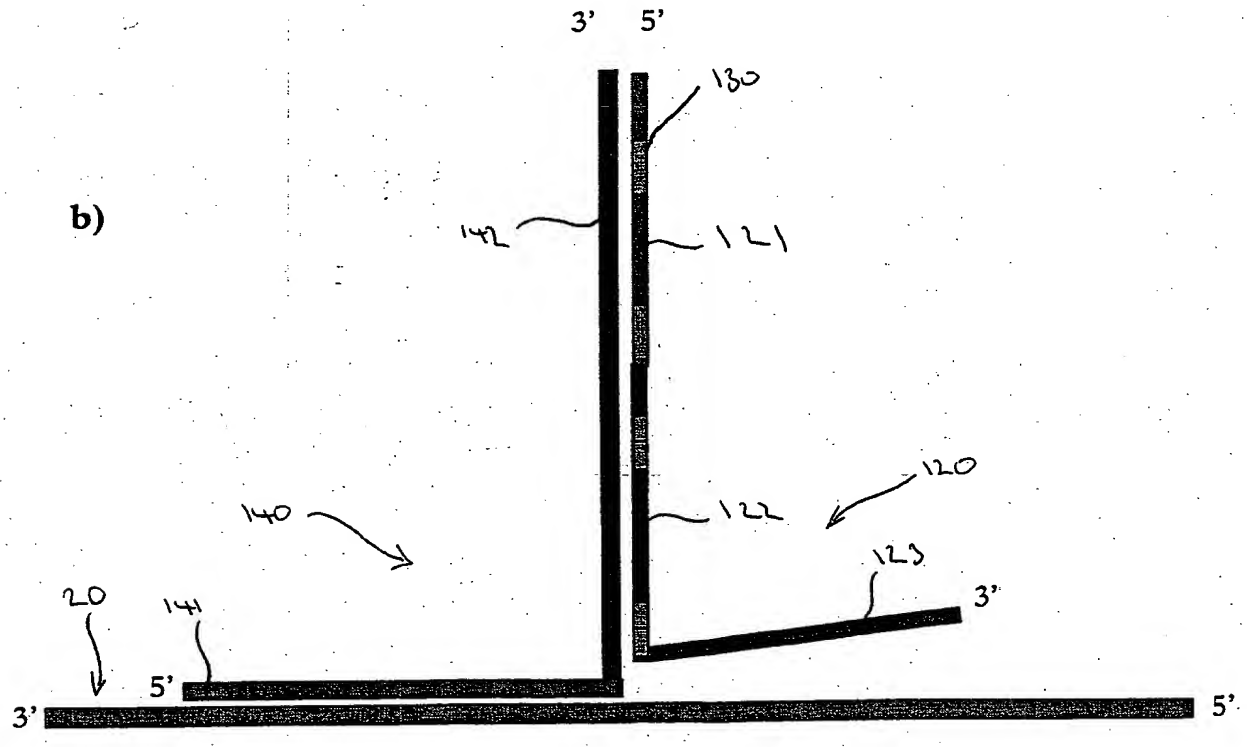
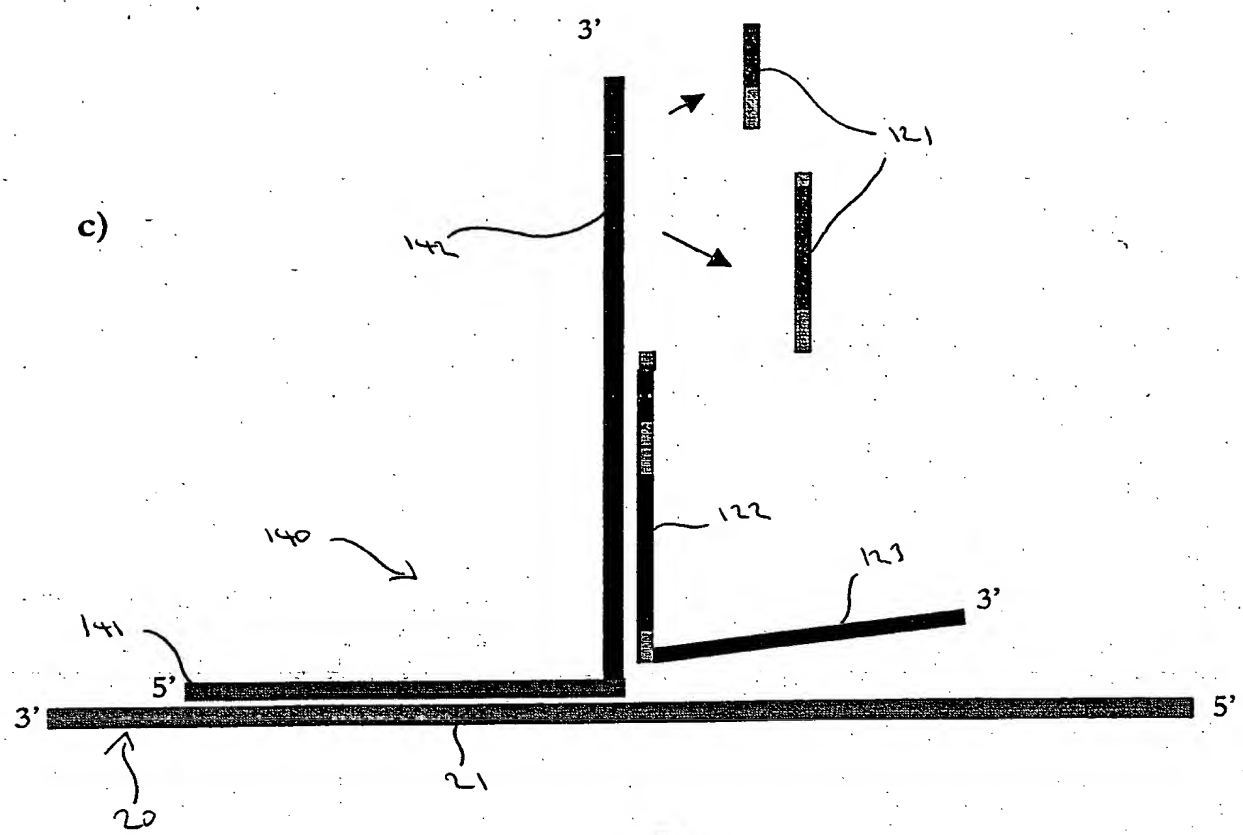


FIG. 8


a)
5'
130
121
130
3'
142
130
120
122
140
123
141
130
3'
5'
3'
5'
20
21


b)
3'
5'
130
142
121
120
140
122
123
20
141
3'
5'
3'
5'


c)
3'
121
142
122
140
123
3'
141
5'
3'
5'
20
21

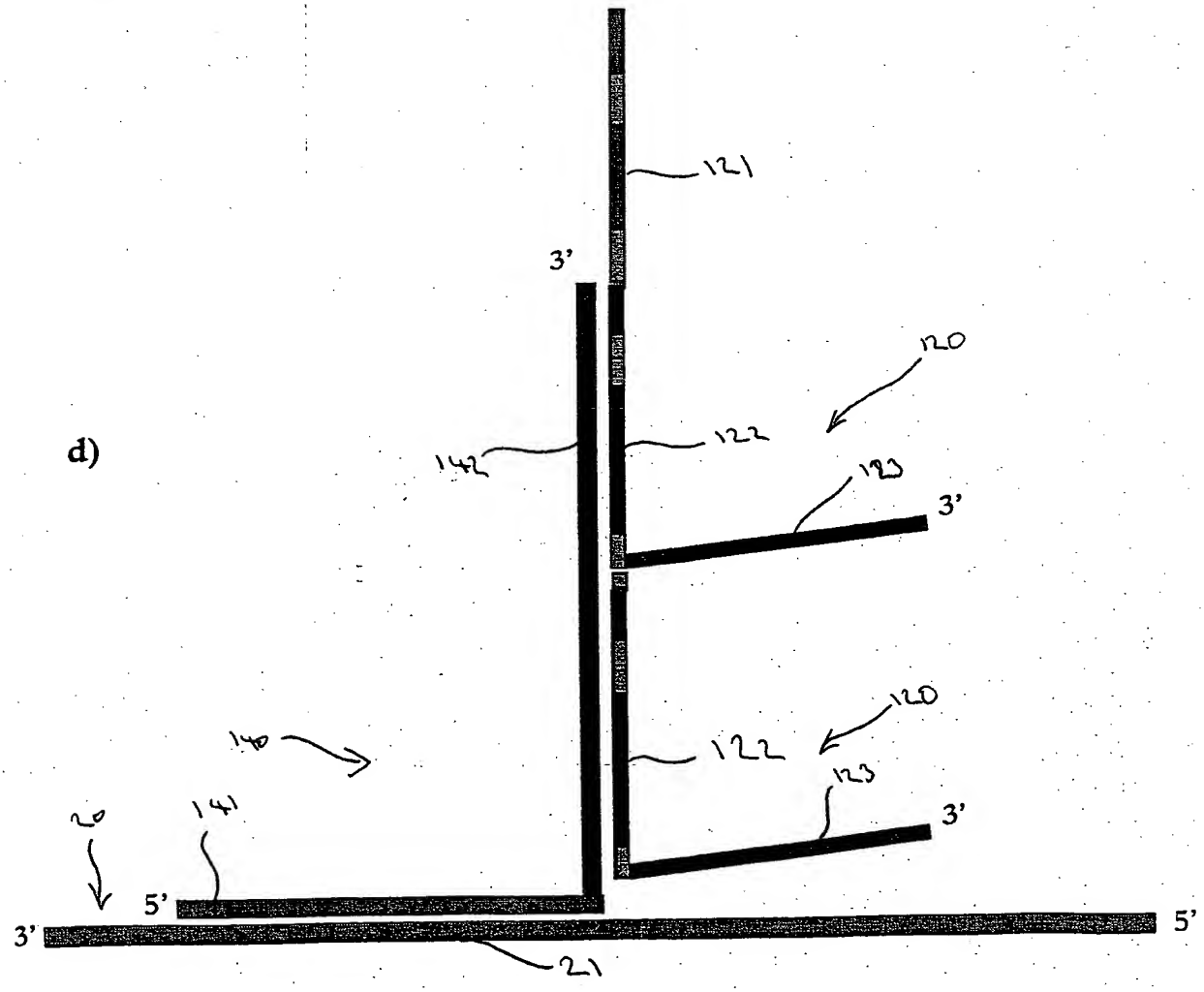
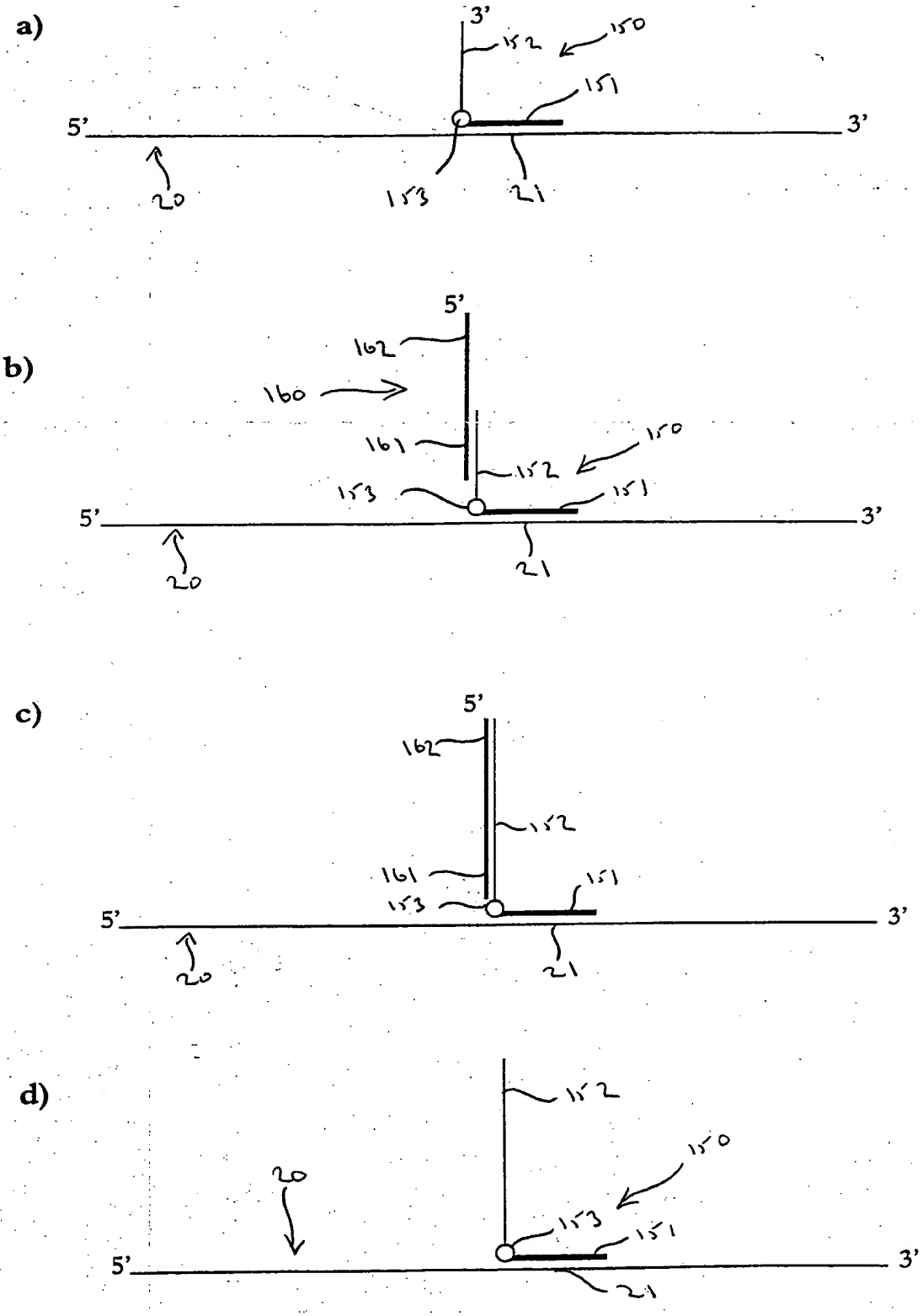
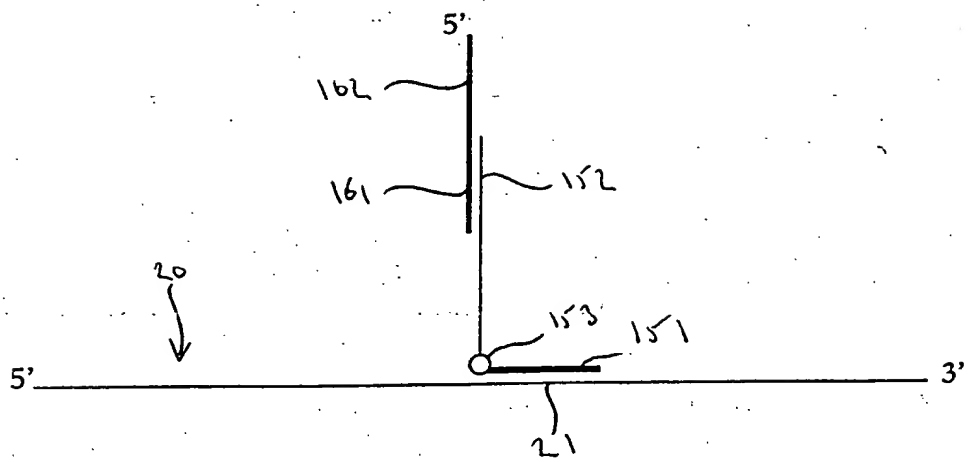

d)
121
3'
120
142
122
123
3'
120
140
122
123
3'
20
141
5'
3'
5'
21

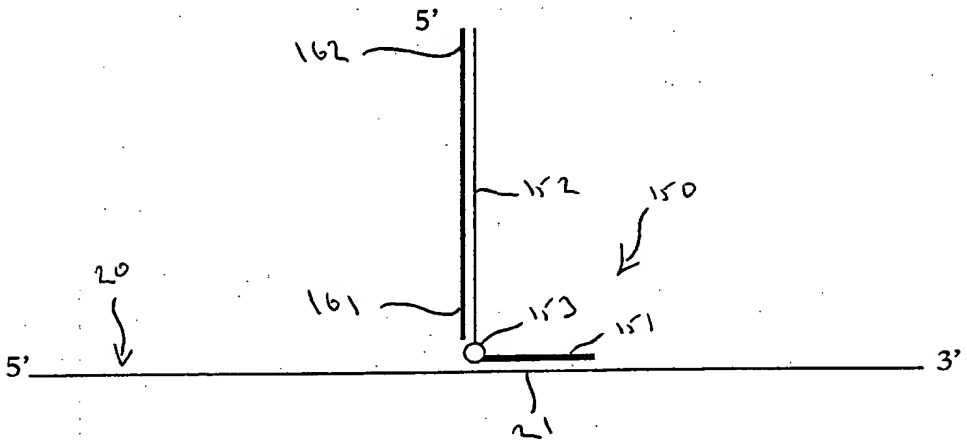
Fig. 9


a)
3'
152
150
151
5'
3'
20
153
21
b)
5'
162
160
161
150
152
153
151
5'
3'
20
21
c)
5'
162
152
161
151
153
5'
3'
20
21
d)
152
150
20
153
151
5'
3'
21

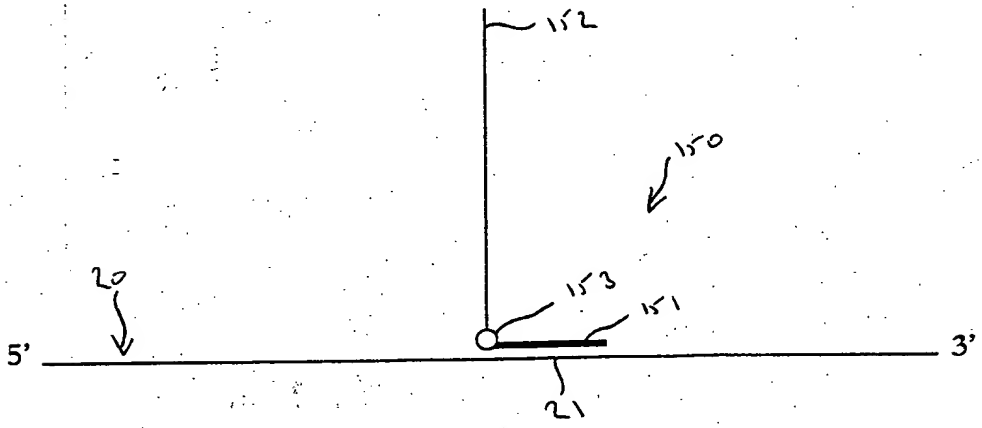
e)


5'
162
161
152
20
153
151
5'
3'
21

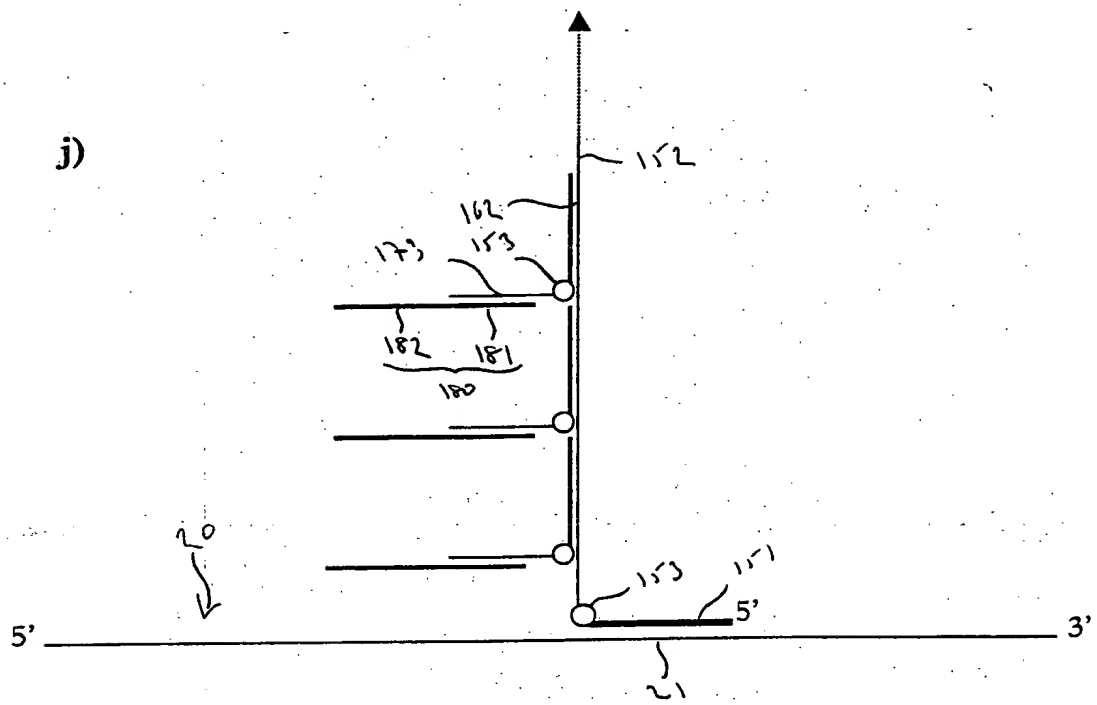
f)


5'
162
152
150
20
161
153
151
5'
3'
21

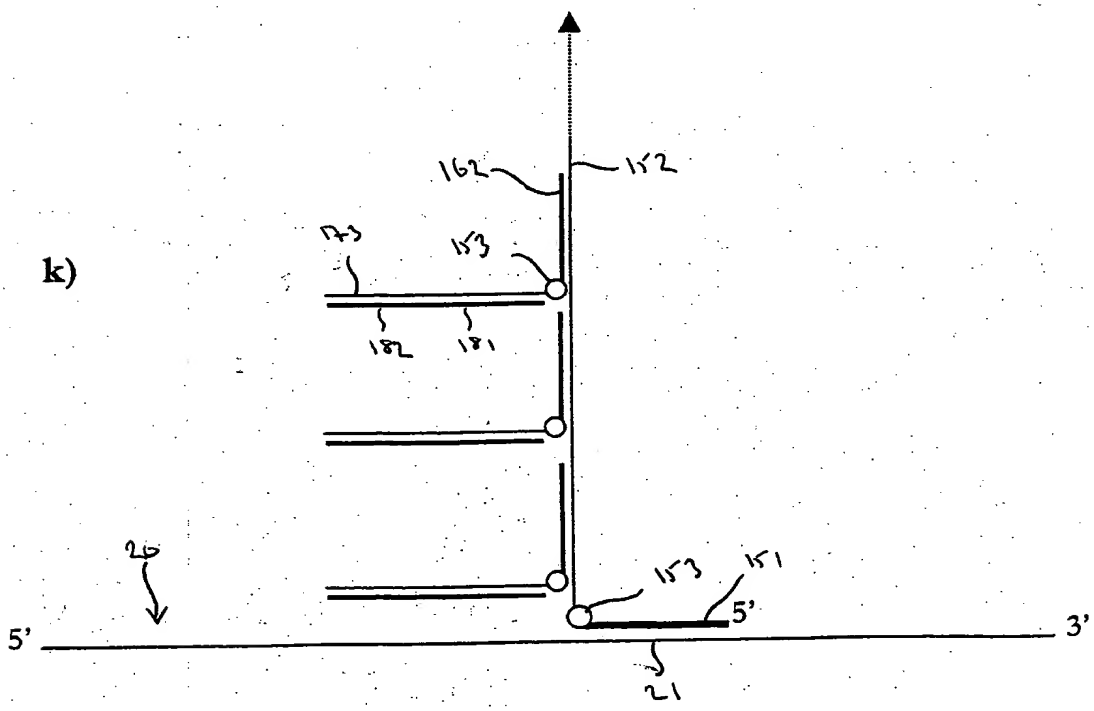
g)


152
150
20
153
151
5'
3'
21

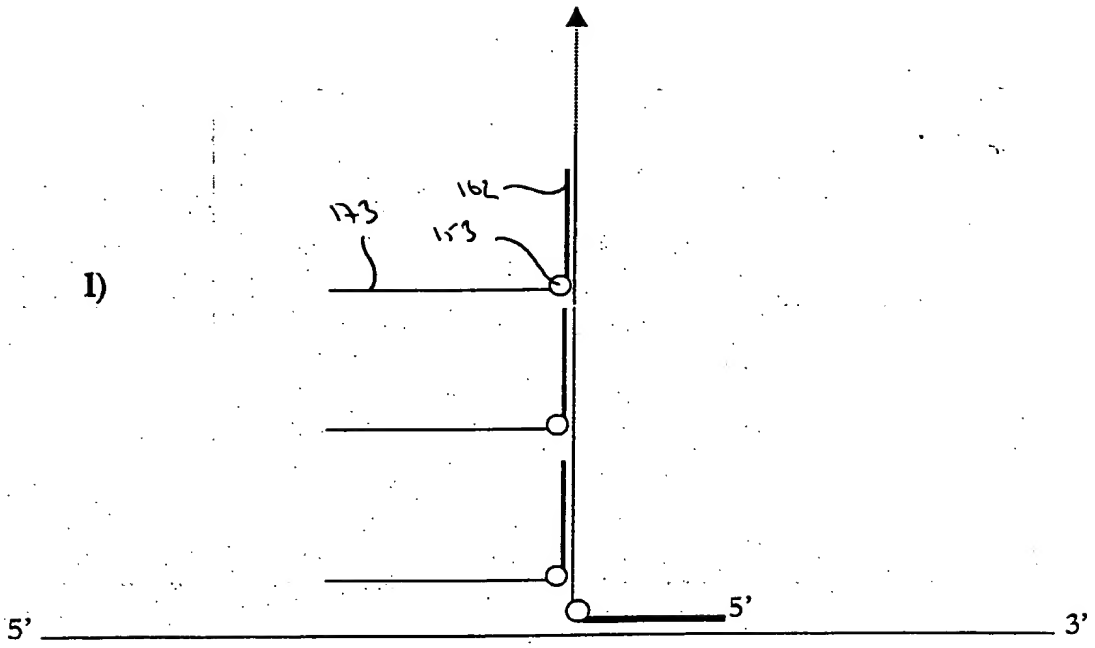
j)


152
162
173
153
182
181
180
20
153
151
5'
3'
21

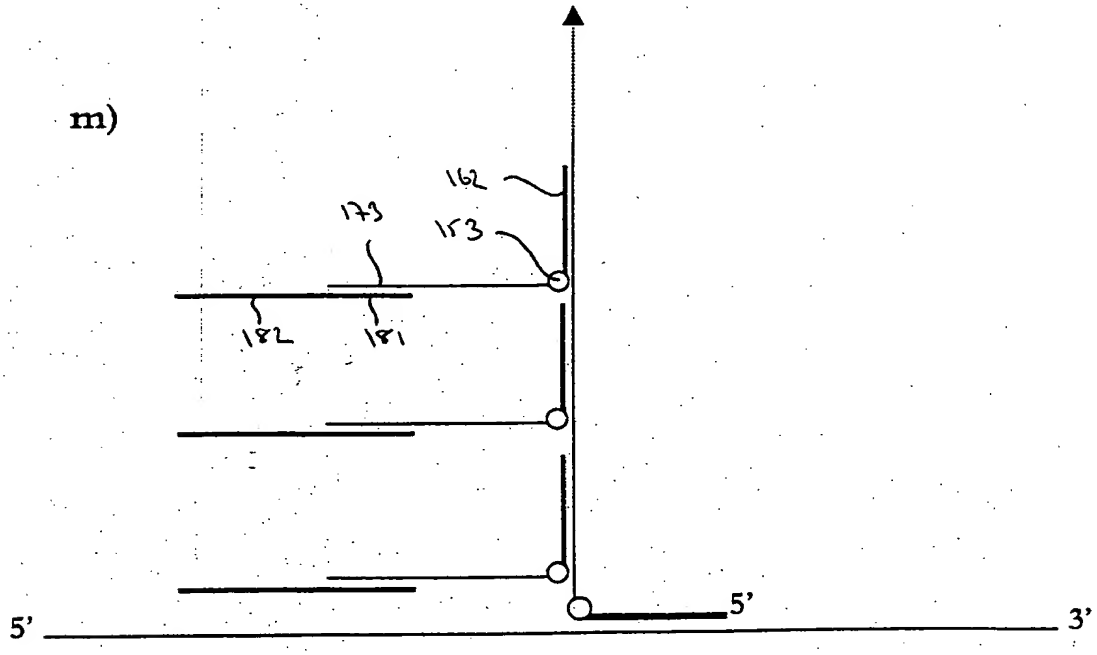
k)


162
152
173
153
182
181
20
153
151
5'
3'
21

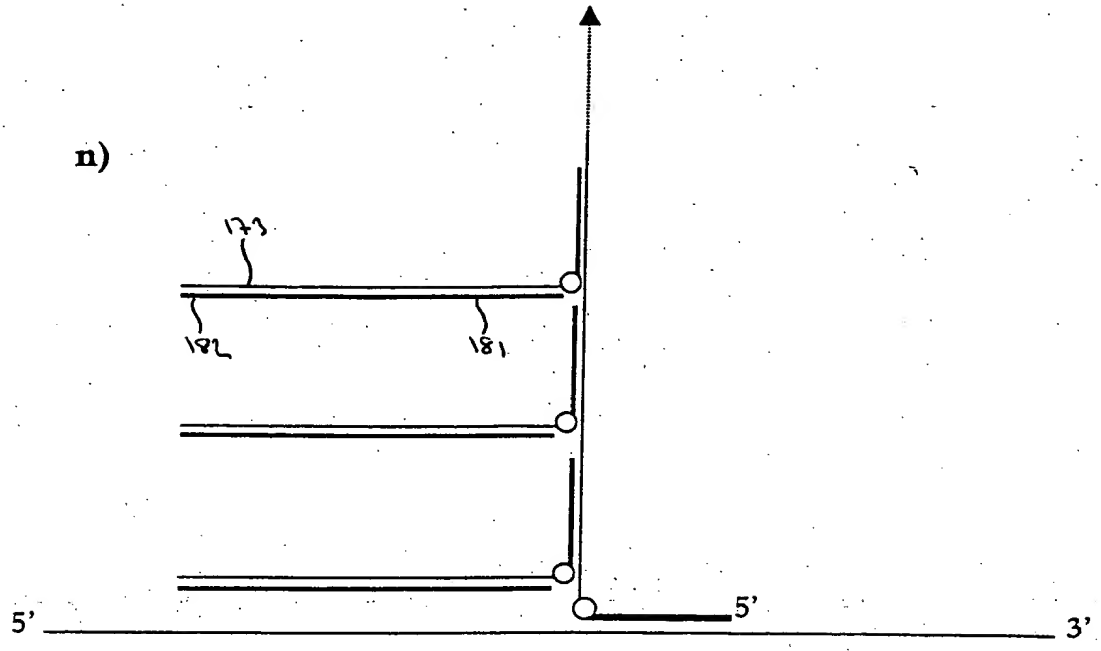
l)



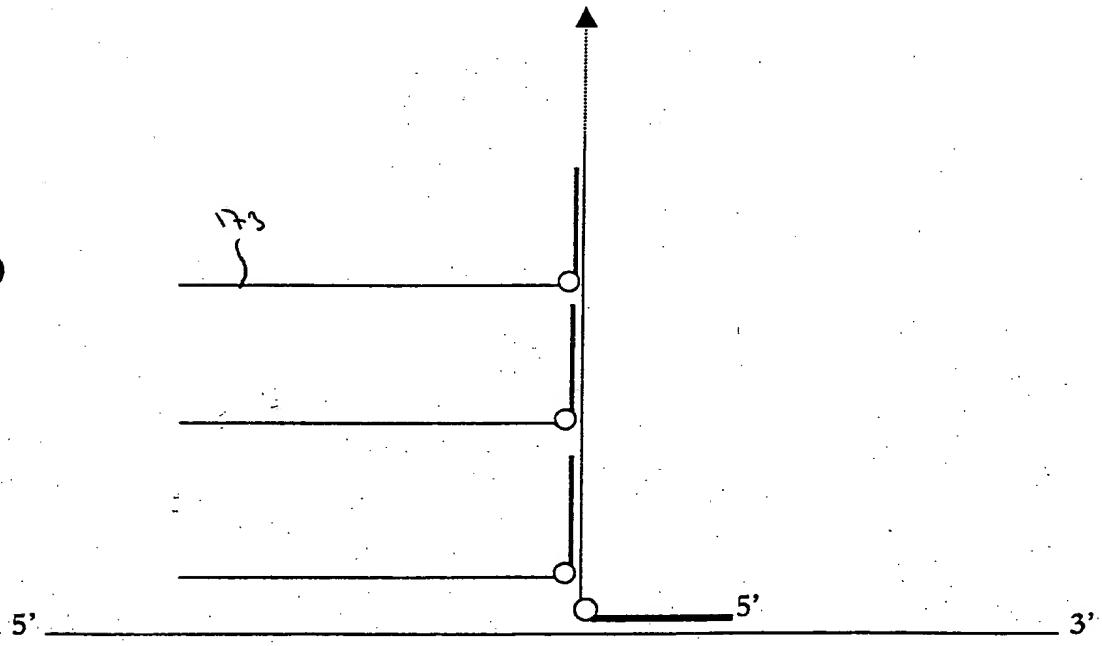
m)



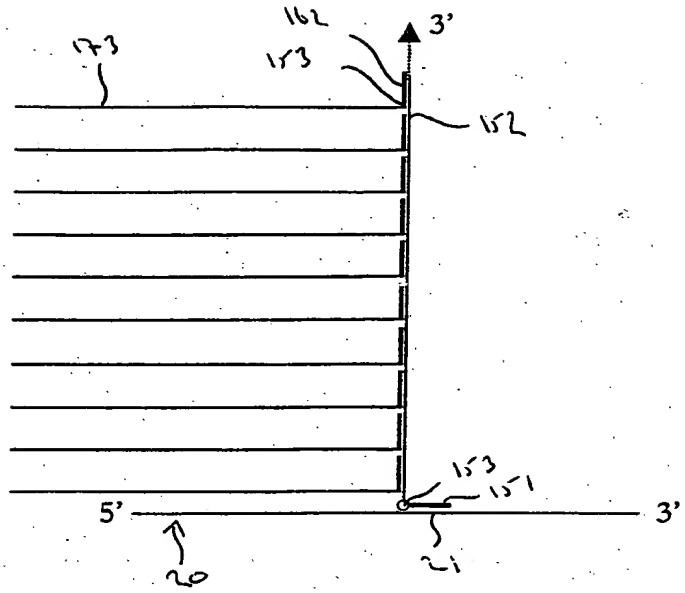
n)



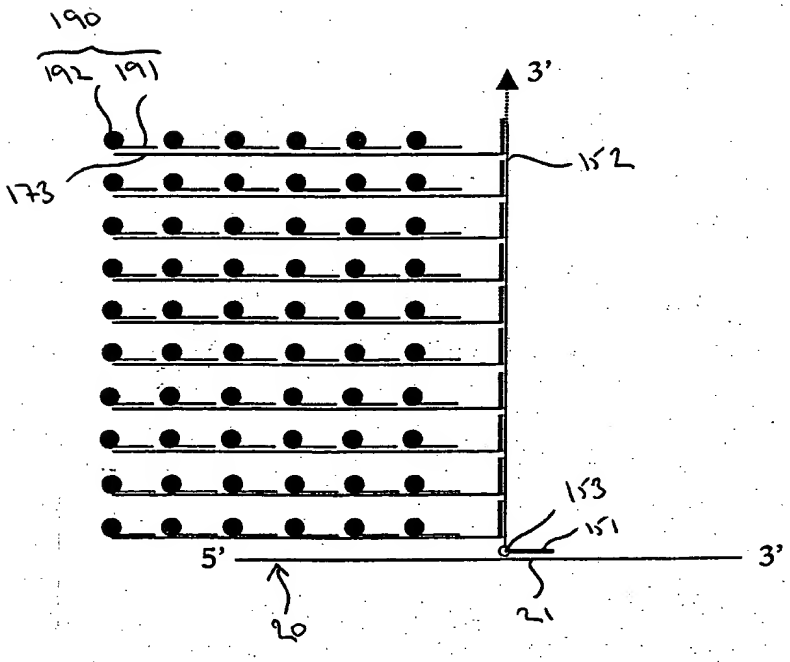
o)



p)


162
3'
173
153
152
153
151
5'
3'
20
21

q)


190
192
191
3'
173
152
153
151
5'
3'
20
21